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It is well established that induction of the p53 tumor suppressor protein in cells can lead to either cell cycle arrest or apoptosis. To further understand features of p53 that contribute to these cell responses several p53-null Saos2 and H1299 cell lines were generated that express wild-type or mutant forms of p53, or the cyclin-dependent kinase inhibitor p21, under a tetracycline regulated promoter. Our results show that while the cellular level of p53 can dictate the response of the cell such that lower levels of p53 result in arrest while higher levels result in apoptosis, nevertheless DNA damage can heighten the apoptotic response to p53 without altering the protein level of p53 in cells. We also demonstrate that arrest and apoptosis are two genetically separable functions of p53 since a transcriptionally incompetent p53 can induce apoptosis but not arrest, while induction of p21, a transcriptional target of p53, can induce arrest but not apoptosis. Finally, we show that a full apoptotic response to p53 requires both its N- and C-termini, and our data suggest that there is synergistic cooperation between transcription-dependent and independent functions of p53 in apoptosis.

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INTRODUCTION

Following genotoxic stress such as that emanating from damaged DNA or conditions of hypoxia, induction of p53 results in cell cycle arrest or apoptosis (reviewed by Levine 1993; Gottlieb and Oren 1996; Ko and Prives 1996). It is not fully understood which of these two responses to p53 is chosen in a given cell. Cell-type appears to be one of the factors (review by Oren 1994) and the microenvironment of a cell can also influence the response (Boudreau et al. 1995; 1996). In addition, several cellular and viral proteins contribute to cells propensity to undergo either response (reviews by Fisher 1994; White 1996). Transformed or tumor-derived cells are frequently more susceptible to apoptosis than their normal counterparts (Lowe et al. 1993).

It is also not fully understood what function(s) of p53 are required for cell cycle arrest or apoptosis. p53 is a sequence specific transcriptional activator of genes which contain p53 response elements (review by Vogelstein and Kinzler 1992). A number of transcriptional targets of p53 have been identified. One of these is the cyclin-dependent kinase inhibitor p21 (El-Diery et al. 1993; Harper et al. 1993; Xiong et al. 1993). Upregulation of p21 expression inhibits the protein kinase activities of G1 cyclin/CDK complexes and thereby prevents phosphorylation of the retinoblastoma (RB) protein (Slobos et al. 1994). p21 is thus an excellent candidate for mediating p53-induced cell cycle arrest. It is not known whether activation of additional targets of p53 are required for arrest as well.

It is less clear how p53 induces apoptosis. There are, however, at least two candidate genes that play roles in apoptosis that can be transactivated in response to p53 induction. In murine cells, p53 upregulates expression of the bax gene (Miyashita and Reed 1995), whose product dimerizes with bcl-2 and prevents the ability of bcl-2 to block apoptosis (Oltvai et al. 1993). It is possible, therefore, that transcriptional activation of the bax gene by p53 induces apoptosis. A second p53 target that might influence apoptosis is IGFBP-3 (Buckbinder et al. 1995) which is an antagonist of insulin-like growth factor-1 (IGF-1) (Baserga 1994). Downregulation of IGF-1 or the IGF-1 receptor is correlated with the apoptotic response (Baserga 1994). Several recent studies have provided evidence, however, that p53 may have a transcription-independent function in apoptosis. p53-dependent apoptosis was shown to occur in cells treated with RNA and protein synthesis inhibitors (Caelles et al. 1994; Wagner et al. 1994). Moreover, transactivation-defective point mutant and truncated p53 proteins can induce apoptosis in HeLa cells (Haupt et al. 1995). However, other experimental results have led to the assumption that transactivation by p53 is, in fact, required for its role in apoptosis in rodent cells (Sabbitini et al. 1995). Thus, different reports have provided essentially contradictory results as to the requirement for the sequence-specific transactivation function of p53 for induction of apoptosis. If indeed there is an alternative function of p53 then it will be important to identify the domain(s) or region(s) of the protein responsible for this function.

p53 has been subjected to extensive analysis of its functional domains (Gottlieb and Oren 1996; Ko and Prives 1996). The p53 polypeptide consists of an activation domain located within the N-terminal 43 amino acids, a sequence specific DNA binding domain located within the central, conserved portion of the protein, and, within the C-terminus resides a tetramerization domain as well as a regulatory region that controls the ability of the protein to allosterically switch from a "latent" form to one that is active for sequence specific DNA binding. Either naturally occurring or experimentally produced mutations within these regions of p53 have identified residues that are important for their function. Mutation of two residues within the p53 activation region, leu22 and trp23, abrogates transactivation by p53 (Lin et al. 1994). This is presumably because these residues are required for the interaction of the activation domain with TATA box binding protein associated factors (TAFs) (Lu and Levine 1995; Thut et al. 1995). The vast majority of the missense mutations that have been detected in tumors of cancer patients map to the central DNA binding domain of p53 (Hollstein et al. 1991).

Among these are a number of mutational "hot spots" that occur with unusually high frequency and together make up about 40% of all tumor derived p53 mutations. Most mutations within the central DNA binding domain impair or abolish sequence-specific DNA binding by p53 (Vogelstein and Kinzler 1992). Finally, mutational analysis of the C-terminus has revealed residues that are important for oligomerization of the p53 protein (Sturzbecher et al. 1992). Moreover, deletion of the C-terminal 30 amino acids of p53 has striking stimulatory effects on the ability of the protein to bind to DNA in vitro (Hupp et al. 1992; Halazonetis and Kandill 1993).

To further understand how p53 might regulate arrest vs. apoptosis we have used the p53 null cell lines Saos-2 and H1299, to generate lines inducibly expressing either wild-type or mutant forms of p53, or wild-type p21. These cell lines have allowed us to (a) identify a quantitative response to different amounts of p53, (b) examine a number of p53 variants in clonally derived cell populations in order to derive information about the influence of functional domains of p53 on cell arrest and apoptosis, and (c) determine the effect of DNA damage on p53 in this context. Our results have both provided new insight into p53 and should provide a paradigm for this approach to studying p53 structure and function, especially in breast cells.

BODY

The level of p53 within Saos-2 cells determines cell death or arrest.

To generate the first series of inducible cell lines, we chose the human osteosarcoma cell line Saos2 because they are null for p53, are easily transfected, and were shown previously to arrest upon overexpression of wild-type p53 (Chen et al. 1990). Each individual cell line used for this study was chosen from a number of clonal lines based on relative levels of protein expressed after induction.

Of the two wild-type p53 inducible cell lines obtained, one of these (p53-7) expressed p53 protein at relatively high levels upon withdrawal of tetracycline and will be refered to as the "high p53 producer" line (Fig. 1A). p53 protein was detected within eight hours and reached maximal levels at 24 hours post-induction (data not shown). Note that the amount of detectable induced p53 protein in p53-7 cells, however, was substantively less than that induced in DNA damaged human RKO cells that contain wild-type p53 (Kastan et al. 1992), demonstrating that the level of p53 expressed in the Saos-2 cell line is within the physiological range. When the growth curve of induced and uninduced p53-7 cells was examined, there was a dramatic difference in cell viability between the two states (Figure 1B). While the uninduced cells continued to grow with a doubling time of ~48 hrs, the p53 expressing cells started to die within 2 days (as determined by reduced cell count) and by 3 days, only 10 % of the cells survived. By 5 days after induction there were virtually no detectable visible cells remaining on the plate (data not shown). The apoptotic cells showed many characteristics of apoptotic process, such as chromosomal condensation and apoptotic bodies. Another characteristic of apoptotic cells is fragmentation of DNA (for review, see Wyllie 1985), and when such cells are fixed in ethanol, DNA fragments leak out of their nuclei. FACS analysis of the DNA content of apoptotic cells indicates that they have less than the normal content of G1 DNA (referred to as sub G1). DNA histogram analysis of induced p53-7 cells (Figure 1C) showed that 1 day after tetracycline withdrawal cells in S phase were reduced from 14 % to 5%, and cells in G2 were increased from 14% to 23%, suggesting that a significant G2 arrest had occurred in at least a fraction of the cells, but at this time point little or no sub-G1 content cells were scored. By 2 days, however, 25% of the cells had a sub-G1 DNA content and at three days after induction more than 60% of the cells had sub-G1 DNA content, with the remaining cells primarily arrested in both G1 and G2. These data suggest that the initial response to induction is cells arrested primarily in G2. With time the accumulation of p53 brings about both arrest in G1 and G2 as well as apoptosis. Virtually all cells die after induction of p53 in p53-7 cells, thus the arrest must be transient since the cells are not protected from eventually entering the apoptotic pathway.

When the second p53 cell line (p53-13) was analyzed, upon withdrawal of tetracycline, the amount of p53 within p53-13 cells was approximately 25-50% of that detected in p53-7 cells (Fig. 2A). However, in contrast with p53-7 cells, although p53-13 cells showed substantially slowed cell growth, there was no reduction in cell number (Fig. 2B) nor appearance of cells with sub-G1 DNA content (Figure 2C). DNA histogram analysis showed that S phase p53-13 cells were reduced from 31% to 15% within 1 day following p53 induction and by 2 to 3 days the proportion of S phase cells remained at that low level (14%) (Fig. 2C). p53-13 cells, therefore, exhibit a significant arrest in both G1 and G2, but no apoptosis. The induction of p53 in both p53-7 and p53-13 cells, notably, was accompanied by a marked increase in the amount of detectable p21 (Fig. 2A).

Since the only obvious difference between the p53-7 and p53-13 cells was the amount of p53 detected after induction, we tested whether the level of p53 was, in fact, capable of regulating the apoptotic vs. arrest response. This was done by varying the amounts of tetracycline in the culture medium of the high producer p53-7 cells. The results showed that low concentrations of tetracycline allowed for a partial expression of p53 while more complete

withdrawal of tetracycline caused greater amounts of p53 to accumulate (Fig. 3A). Cells were counted at day 0, 1, or 3 following induction of p53 to different extents. The number of surviving cells was generally both inversely proportional to the amount of p53 expressed and directly proportional to the amount of tetracycline present in the culture media (Fig. 3B). With the conditions resulting in the two lowest amounts of p53 induced (i.e. 40 and 20 ng/ml of tetracycline), however, there was evidence of some increase in cell number, albeit significantly less than with no induction. Thus, intermediate levels of p53 caused slowed cell growth and cell arrest but not a significant amount of cell death, while levels close to or the same as that seen with full induction caused comparable loss of cells to that shown in Figure 1. We conclude from these data that the level of p53 in the p53-7 Saos 2 cell line can determine whether the cells undergo growth arrest or apoptosis.

DNA damage can sensitize cells to p53-mediated apoptosis without affecting the level of p53 protein.

Effectors of DNA damage have been shown to increase the amount of p53 in cells by a post-transcriptional mechanism (Maltzman and Czyzyk 1984; Kastan et al. 1992; Lu and Lane 1993). Since the quantity of p53 induced was clearly a determinant of the switch between arrest and apoptosis in p53-7 cells, we wished to test whether the levels of p53 in the low producer cell line, p53-13, could be augmented after DNA damage, and whether the cells would now undergo apoptosis. Camptothecin (CPT), a topoisomerase inhibitor and cancer therapy drug, has been shown to induce DNA damage in cells (Nelson and Kastan, 1994). Moreover, as shown in Fig. 1A, we have confirmed that treatment of RKO cells with CPT results in a significant induction of p53 protein levels. When p53-13 cells were treated with increasing amounts of CPT in the presence or absence of tetracycline we observed that even without p53 induction there was a modest apoptotic response to CPT suggesting that Saos-2 cells can undergo DNA damage associated apoptosis in a p53- independent manner (Fig. 4B). Unexpectedly, however, when p53 was induced in CPT-treated p53-13 cells there was both a significant increase in the number of apoptotic cells (Fig. 4B), and yet no discernable increase of p53 protein levels (Fig. 4A). Thus p53 and CPT cooperate in Saos2 cells to cause a strong apoptotic response, and this occurs in a manner that is independent of p53 protein accumulation. Furthermore, our preliminary results showed that CPT does not elevate the extent of apoptosis induced by transactivation-deficient p53 (gln22/ser23) in Saos-2 cells (data not shown).

p21 induction in Saos2 cells leads to arrest but not apoptosis.

The cyclin-dependent kinase inhibitor, p21 (WAF1), a potential mediator of p53 tumor suppression, has been shown in a number of studies to be strongly induced by p53. Consistent with these results p21 expression was markedly increased in both p53-13 and p53-7 cells after removal of tetracycline (Fig. 2A). Since the low p53 producer cells (p53-13) did not undergo apoptosis this suggested that p21 induction alone is not sufficient for apoptosis. To directly analyze the role of p21 in cell death or arrest, we examined the cellular response to induction of p21 in the absence of p53. One of the p21-inducible cell lines (p21S4) expressed p21 protein to an even greater extent than in induced p53-7 cells (Fig. 5A). However, upon maximal p21 induction, p21S4 cells underwent cell cycle arrest with no evidence of apoptosis after induction (Fig. 5B). DNA histogram analysis showed that in the p21S4 line S phase cells were reduced from 32% to 14% following 3 days of p21 induction and that cells were arrested in both G1 and G2 (Fig. 5C). It is notable that both the kinetics of cell growth and DNA histogram analysis of p21S4 and p53-13 cells were very similar after removal of tetracycline (compare Fig 2B. with Fig. 5B). Our data thus strongly suggest that in Saos2 cells growth arrest by p53 is mediated by p21, but even when expressed at very high levels p21 on its own is unable to produce an apoptotic response.

A weak and delayed apoptotic response is induced by a transactivation-deficientmutant form of p53.

A p53 double mutant (gln22/ser23) was shown to be defective in transactivation (Lin et al. 1993) presumably due to the inability of this mutant to bind to TAFs that are critical for p53 mediated activation (Lu and Levine 1995; Thut et al. 1995). This mutant, however, has produced contradictory results as to whether transcriptional activation is necessary for p53-mediated apoptosis (Haupt et al. 1995; Sabbitini et al. 1995). To determine whether apoptosis can occur through a p53 transactivation-independent pathway in Saos-2 cells, a cell line, 22/23-4, which expresses high levels of inducible transactivation deficient p53 (gln22/ser23) was used. Consistent with evidence from in vitro studies (Lin et al. 1994) and transient transfections (Lin et al. 1995), this mutant form of p53 was transcriptionally inert since endogenous p21 was not induced even by high levels of p53 (gln22/ser23) (data not shown). Consistent with the lack of induction of p21 in 22/23-4 cells, no detectable cell cycle arrest was observed after induction of mutant p53 as observed by DNA histogram analysis (Fig. 6). Nevertheless, in these cells p53 (gln22/ser23) induced cell death (Fig. 6), although to a lesser extent and with delayed kinetics as compared to wild-type p53. Thus, those cells which did not undergo apoptosis contained a normal S-phase DNA content and presumably kept cycling. The apoptosis induced by p53 (gln22/ser23), although reduced, was significantly greater than either the background levels of cell death that occur in the presence of tetracycline, or than in cell expressing mutants completely defective in apoptosis (see Fig. 8 for comparison). It is possible that the induction of apoptosis in the absence of induction of p53 may be explained by basal (or leakiness) level of p53 expression. Our data confirm and extend observations by Oren and colleagues (Haupt et al. 1995) who showed that apoptosis can be brought about by p53 mutants such as p53 (gln22/ser23) that are defective in sequence specific transactivation in transiently transfected HeLa cells. These data also provide clear evidence that the abilities of p53 to induce cell cycle arrest and apoptosis are genetically separable.

The p53 C-terminus is necessary for efficient apoptosis.

p53 contains an autoinhibitory region within the last 30 amino acids of the protein. Deletion of this region generates a p53 protein that is activated for DNA binding in vitro (Hupp et al. 1992) and that is comparable to full length p53 in activating transcription in transient transfection assays in cells (Halazonetis and Kandil 1993; L. Ko unpublished observations). To determine the cellular response to a p53 lacking the C-terminal 30 amino acids (p53ΔC30), cell lines expressing this p53 variant were isolated. One of these lines, p53ΔC30-6, contained at least two-fold more p53 than the high producer cell line p53-7 when normalized to the cellular actin protein levels (Fig. 7A). Consistent with observations that the ΔC30 mutant is functional in transactivation, p21 was induced in p53 (ΔC30) cells to a similar extent as that by wild-type p53 (Fig. 7B). Unexpectedly, however, although the growth of p53ΔC30-6 cells was completely arrested upon induction, the cell number did not decrease detectably throughout the time course of the experiment (Fig. 7C). Since very slow but detectable cell growth had been observed for both the low p53 producer cell line p53-13 (Fig. 2B) and high p21 producer cell line p21S4 (Fig. 5B), and both cell lines do not undergo apoptosis, the flat growth curve of ΔC30-6 cells suggested the possibility that a minor proportion of ΔC30-6 cells underwent apoptosis. Consistent with this idea, DNA histogram analysis of ΔC30-6 cells taken over 4 days showed that upon induction of p53ΔC30, a weak apoptotic response with delayed kinetics ensued (Fig. 7D). Therefore, in this cell line p53 (ΔC30) can induce apoptosis, but far more weakly than full-length p53, suggesting that the C-terminal 30 amino acids of p53 are required for efficient apoptotic activity. We considered the possibility that the ΔC30-6 cells containing inducible p53ΔC30 had become intrinsically defective for p53 mediated apoptosis. However two points argue strongly against this. First, as seen with the low producer wild-type p53 cell line, p53-13, treating p53ΔC30-6 cells with camptothecin led to a marked increase in cell death (data not shown). Second, additional Saos2 and H1299 cell lines expressing p53ΔC30, were all similarly

defective in apoptosis (see below). We therefore conclude that in Saos2 and H1299 cells a strong apoptotic response requires the intact C-terminus of p53.

A full apoptotic response to p53 in tumor cells requires both sequence-specific transactivation and C-terminal regulatory domains of p53

To gain further information as to whether the C-terminal domain of p53 would itself be sufficient to induce apoptosis in the absence of the N-terminus, a Saos-2 cell line ΔN96-5 expressing amino acids 97 to 393 [p53 (ΔN96)] was used. ΔN96-5, which lacks the entire transactivation domain, is capable of sequence specific DNA binding (our unpublished observations) but is completely defective in SST (Pietenpol et al. 1994). Upon withdrawl of tetracycline, ΔN96-5 cells expressed markedly higher levels of truncated p53 than in the high producer wild-type p53 cell line, p53-7 (data not shown). Yet induction of p53ΔN96 had no measurable effect on cell growth or arrest (Table 1 and Fig. 8). This indicates that neither DNA binding nor the C-terminal domain are sufficient for apoptosis.

Our results show that p53 with mutations within the N- and C- termini were inefficient but not completely inert in inducing apoptosis in Saos-2 cells. Tumor-derived mutant forms of p53 contain intact N- and C-termini, but are incapable of binding specifically to p53 responsive elements and of transactivation (for reviews, see Gottlieb and Oren 1996; Ko and Prives 1996). In order to determine if such mutants would display any apoptotic activity in Saos-2 cells, cell lines which contained inducible mutant forms of p53 (ser249 or his175) were generated. Upon induction of high levels of either p53 (ser249) or p53 (his175), essentially no changes in growth or survival were detected in the Saos-2 cells as compared to either the uninduced state or the parental cell from which they were derived (Table 1 and Fig. 8). These results confirm that tumor derived mutant forms of p53 are inert for inducing the apoptotic response. Note that although we show here only data for hot-spot mutant p53 proteins p53(his175) and p53(ser249), we have observed that several additional tumor derived mutants are unable to induce apoptosis as well (Friedlander et al. in press and our unpublished observations).

Levels of bcl-2 and bax within Saos-2 cells are not affected by strong induction of p53 leading to apoptosis.

The bax polypeptide forms a heterodimer with bcl-2 and blocks the ability of the latter to inhibit apoptosis (for review, see White 1996). An increased ratio of bax to bcl-2 is known to accelerate the apoptotic response. In rodent cells, the temperature-sensitive murine p53 (ala135) stimulates bax but inhibits bcl-2 expression at permisive temperature when it is in the wild-type conformation (Miyashita et al. 1994). Thus, it has been hypothesized that the bcl-2 protein family mediates p53-dependent apoptosis. Indeed, the bax gene contains a p53 responsive element, suggesting that bax is a direct transcriptional target of p53 (Miyashita and Reed 1995). To determine whether the ratio of bax to bcl-2 is increased by the induction of p53 in Saos-2 cells, levels of both bax and bcl-2 were quantitated by western blot. Upon full induction of p53 in high producer cells and ensuing apoptosis, bax expression was not increased and bcl-2 expression was not inhibited (Fig. 9). Thus, there was no change in either the levels or the ratio of bax and bcl2 polypeptides in p53-7 cells, suggesting that other transcriptional targets of p53 must be involved as well.

H1299 cells with inducible wild-type and mutant forms of p53 confirm and extend results in Saos2 cells

During the course of the experiments described above with Saos2 cells it was decided to use the same strategy to generate a number of additional inducible cell lines in another p53-null human cell background. We chose H1299 cells because, although like Saos-2 osteosarcoma cells they are easily transfectable and can undergo p53-mediated apoptosis, they are of a

different cellular origin (small cell lung carcinoma) and, importantly, in contrast to Saos-2 cells, they express the RB tumor suppressor protein (data not shown). Since a relationship between RB and p53 has been established in many experimental models (for review, see White 1996) it was of interest to determine the response of these cells to the different forms of p53 that were tested in inducible Saos-2 cells. The results of our experiments are summarized in Table 1. As was observed with Saos2 cells, one high p53 producer H1299 cell line underwent apoptosis after induction while a low producer H1299 cell line underwent arrest. However, the high producer H1299 cells displayed a more rapid and extensive apoptotic response than seen with the Saos2 high producer cell line. The increased kinetics seen with this cell line may be due to the faster doubling time of H1299 cells (eg. 24 h) as compared to Saos-2 cells (eg. 48 h). Consistent with results observed in Saos2 cells, H1299 lines expressing ser249, his175 or ΔN96 mutant forms of p53 were completely unable to induce apoptosis (Table 1). Moreover, cells expressing the C terminally truncated mutant p53ΔC30 or the transactivation defective mutant p53 (gln22/ser23), underwent apoptosis with reduced kinetics and extent over the time course examined. Importantly, a p53 variant that contained both the N-terminal double mutation at residues 22 and 23 but which also lacked the C-terminal 30 amino acids, although also expressed at high levels in H1299 cells, was completely inert in inducing apoptosis or growth arrest. This finding provided the strongest evidence that both N- and C-termini of p53 are required for apoptosis in tumor cells.

CONCLUSION

Inducible cell lines provide insight into p53 responses in tumor cells.

The cell lines described herein have provided several novel observations about the cellular response to p53. We show for the first time that within a given clonal cell line the level of p53 can determine whether cells arrest or die. We also demonstrate that although DNA damage can cooperate with p53 to elicit an apoptotic response, this occurs without detectable alteration in the amount of the p53 protein. Furthermore, our results show that the arrest and apoptotic responses are genetically separable activities of p53. Finally, our data suggest that the p53 protein has multiple domains that function in inducing cell death and that these domains cooperate synergistically to produce a full apoptotic response.

The use of tetracycline regulated promoters to study p53 and p21 has been described previously. Chen et al (1995) showed that induction of p21 by withdrawl of tetracycline can suppress cell proliferation as well as tumorigenicity. However this study did not address whether cells overexpressing p21 underwent arrest or apoptosis. Agarwal et al (1995) generated a tetracycline regulated p53 cell line in MDAHO41 fibroblasts, and showed that induction of p53 led to arrest at both G1 and G2 with data similar to our results with low producer Saos2 and H1299 cells. This system has also been used to identify p53 responsive genes (Buckbinder et al 1995), as well as to show that expression of p53 in glioblastoma cells leads to the secretion of an inhibitor of angiogenesis (Van Meir et al. 1994). One drawback of clonally derived cell lines, however, is the limited number of lines from which conclusions can be drawn. Whenever possible we confirmed that observations made for each p53 variant were reproducible for additional isolated lines (data not shown). Although the results obtained are representative of Saos2 and H1299 cells, the observations we make are not necessarily generalizable to all other cells. While our results may not be fully informative with regard to the function of p53 in normal diploid cells, they may have a more practical relevance. There is good evidence that primary cells are less prone to apoptosis by p53 than are cells derived from tumors (Lowe et al. 1993). The greater propensity of tumor cells to undergo apoptosis might be important for the design of therapeutic reagents that would increase the magnitude of a p53 response in tumor cells.

The role of p53 as a transcriptional regulator in arrest and apoptosis.

The response of Saos2 cells to the induction of p53 was dictated by the quantities of protein produced. When levels of p53 were lower, cells showed slowed or arrested growth, while at higher levels of p53, cell death ensued. As a transcriptional regulator, p53 binds to its cognate sites in p53 responsive genes and activates transcription of those genes. Both the human and the mouse p21 promoters contain two separated p53 binding sites (El-Diery et al. 1993; Macleod et al. 1995). Looping of DNA mediated through interaction of p53 bound at both sites has been demonstrated to facilitate transactivation by p53 (Wang et al. 1995). Because of this configuration of cooperating sites, one might predict that p21 would require relatively little p53 for optimal activation. Indeed, our preliminary results suggest that maximal activation of p21 in p53-7 cells is obtained even with very low levels of p53 (data not shown) and thus, very little p53 is required to drive expression of p21 and consequently to effect growth arrest.

It is likely that p53 transactivation also contributes to the apoptotic response since the cell death induced in response to the transactivation defective p53 (p53gln22/ser23) occurs with delayed and reduced kinetics as compared to that seen with wild-type p53. p53(ΔC30), which lacks the C-terminal 30 amino acids regulatory domain but has comparable ability to activate transcription, also induces a weak and delayed apoptosis. However, the doubly altered mutant p53(gln22/ser23ΔC30) is inert for such activity (Table 1). Therefore, our data imply that while

the ability of p53(gln22/ser23) to induce apoptosis is transactivation-independent, that of p53(ΔC30) is transactivation-dependent, highlighting the fact that p53 acts to induce apoptosis by at least two discrete pathways.

Since p21 induction is not correlated with p53-mediated apoptosis in the cell lines examined, there may be alternate p53 target genes activated at higher levels of p53, whose expression is necessary for apoptosis. We propose that such gene(s) might be bound relatively weakly by p53 and thus would require more p53 protein to ensure sufficient site occupancy for transcriptional activation. Interestingly, tumor-derived mutant forms of p53(ala143) (Friedlander et al. 1996) or p53 (pro175) (Ludwig et al. 1996) are defective in inducing apoptosis but can induce transcription from a limited subset of p53 responsive elements. These mutants can activate transcription from promoters with responsive elements from p21, mdm-2 and cyclin G, but not bax or IGFBP3 genes. Notably, wild-type p53 binds to the bax and IGFBP3 cognate sites relatively poorly, consistent with our proposal that p53 target genes involved in apoptosis may have weaker affinity binding sites. Although the bax gene is an obvious candidate, induction of bax was not observed in Saos2 cells. This implies that there are one (or more) p53 responsive gene other than bax which p53 activates in these cells. We are currently examining whether other candidate target genes such as IGFBP3 are activated by p53 in these inducible cell lines.

The role of p21 in arrest and apoptosis.

Cell lines expressing p21 underwent arrest but not apoptosis in the absence of p53. The growth curves and FACS profiles of cells expressing p21 were similar to those expressing lower levels of p53 (compare Fig. 2 and 5). In each case a dramatically reduced growth rate was accompanied by arrest in both G1 and G2. While we cannot rule out that other targets of p53 may also be involved, these data imply that the arrest response of Saos2 and H1299 cells to moderate levels of p53 is caused primarily by induction of p21. It is also clear that p21 induction in these cells is insufficient to induce apoptosis. This conclusion is derived from the following results: (1) high levels of p21 expressed either with or without p53 did not cause apoptosis in Saos2 cells; (2) the p53ΔC30 cell line which shows a very reduced apoptotic response is as effective as wild-type p53 in inducing p21 and cell cycle arrest; and (3) the transcriptionally defective p53 mutant [p53(gln22/ser23)] can not induce p21 (nor can it effect a cell cycle arrest) and yet it can induce apoptosis, albeit to a lesser extent.

Despite the fact that p21 was induced to comparable levels in p21- and p53- inducible cell lines, the high producer p53-7 cells underwent both arrest and apoptosis. The fact that apoptosis was the eventual fate of virtually all p53-7 cells indicates that the p21-mediated arrest in these cells is not sufficient to protect the cells from the death response.

Speculation on a transcription-independent role for p53 in apoptosis.

Given data from previous studies as well as the data presented here, it is clear that p53 can induce apoptosis without transcriptional activation. The magnitude of the apoptotic response varied dramatically with the p53 mutant that was induced (Fig. 8 and Table 1). Since the extent and kinetics of apoptosis induced by intact wild-type p53 are far greater than those by either of p53 (ΔC30) or p53 (gln22/ser23), we propose that transcription dependent and independent apoptotic pathways induced by these p53 variants, respectively, synergistically cooperate to induce a full apoptotic response. Cells expressing p53 (ΔN96) which lacks the N-terminal 96 amino acids but has an intact C-terminus can not induce apoptosis. Taken together with the results of the p53Δ30 mutant we conclude that both N- and C-termini must be intact in order to produce a strong p53 apoptotic response. Again, the fact that a mutant p53 with both a mutated N-terminus and truncated C-terminus [p53(gln22/ser23ΔC30)] is absolutely inert for both apoptosis and arrest in H1299 cells underscores this conclusion. Paradoxically, however,

tumor derived mutants which contain intact N- and C-termini are also completely inert for inducing apoptosis. It is well established that the one feature common to the tumor-derived p53 mutations is a defect in sequence specific p53 DNA binding. Therefore, our results suggest that p53 must be bound to cognate sites in DNA but not necessarily activating transcription in order for it to be in the correct confirmation for its role in apoptosis.

To explain our results, we propose the following model (Fig. 10): Interactions with a bi- or multi-component factor would be required to associate with regions both at N- and C-termini of p53, when it is bound to DNA, in order to cause apoptosis. While the N-terminal 22 amino acids of p53 would be dispensable for its interaction with the hypothetical factor at both ends (eg. p53ΔN22), deletion of the entire N-terminus (eg.p53ΔN96) would completely abrogate its interaction with the factor at the N-terminus, rendering this truncation of p53 incapable of inducing apoptosis. By contrast, mutations affecting part, but not all, of the N- and C- termini, such as p53(gln22/ser23) and p53ΔC30, would diminish but not completely destabilize interaction with the putative multi-protein complex, resulting in a lesser degree of apoptosis. Finally, N- and C terminal double mutations p53 (eg. gln22/ser23ΔC30) would completely disrupt complex formation so as to preclude an apoptotic response. In this model, massive over-expression of either the N-terminus alone or C-terminus alone would be predicted to induce apoptosis to some extent, a suggestion that is borne out by results showing that overexpressed p53 N-terminal fragment 1-214 (Haupt et al. 1995) or C-terminal fragment 319-393 (Wang et al. 1996) can induce some degree of cell death. A number of proteins have been reported to interact with p53 in vitro and in vivo. Of particular relevance to p53's function as a transcriptional regulator is its interaction with the basal transcription factors TFIID and TFIIH, both of which contain more than one polypeptide component that was reported to interact with p53. In each case these interactions involve both N- and C-termini of p53 (Xiao et al. 1994; Horikoshi et al. 1995; Wang et al. 1995). The TFIID component TATA binding protein (TBP) was reported to bind both the N- (Seto et al. 1992; Liu et al. 1993) and the C-termini (Horikoshi et al. 1995), while the TAFs, Drosophila TAFs 40 and 60 (Thut et al. 1995) and the human TAF30 (Lu and Levine 1995), were shown to interact with only the p53 N-terminus. With respect to TFIIH, the p62 polypeptide subunit has been shown to interact with the N-terminus (Leveillard et al. 1996), while the ERCC2/XP-B and ERCC3/XP-D polypeptide components were shown to bind to the p53 C-terminus (Wang et al. 1995; Leveillard et al. 1996). Interaction of p53 with TFIID or TFIIH would most likely be facilitated when p53 were bound to DNA. Indeed we previously reported cooperative interaction between p53 and TFIID when both were bound to DNA (Chen et al. 1993). It is of course also possible that other cellular factors might be involved. Cellular proteins of potential interest that were reported to interact with p53 include mdm-2 (Momand et al. 1992; Oliner et al. 1993), the nuclear proto oncogene c-abl (Goga et al. 1995), the single stranded binding protein replication factor A (RP-A) (Dutta et al. 1993; He et al. 1993; Li and Botchan 1993), Wilms tumor suppressor protein (WT-1) (Maheswaran et al. 1995), and transcription activators Sp1 (Borellini and Glazer 1993) and CBF (Agoff et al. 1993). Any of these and other as yet unidentified proteins may be involved in the initiation of the pathway toward apoptosis in response to p53.

p53 expressing cells are more sensitive to apoptosis when DNA damaged

Even though a low level of p53 is not sufficient to induce apoptosis in Saos-2 cells, it can sensitize cells to undergo apoptosis following CPT-induced DNA damage. Because calcium phosphate-mediated DNA transfection of cells can induce p53 and an ensuing growth arrest (Renzing and Lane 1995), this procedure might also cooperate with expressed p53 to amplify the apoptotic response in transient transfection assays. This may explain why our experiments show a much weaker and delayed cell death for the p53 (gln22/ser23) cell lines than previously reported when transient transfection assays were employed (Haupt et al. 1995). How these two agents, DNA damage and p53, cooperate is a matter of great interest. While there is considerable evidence that p53 is stabilized after DNA damage, it has been speculated that DNA damage

might also convert p53 to a more active DNA binding state (Lu and Lane 1994). This intriguing possibility is currently under investigation. We are excited by the potential of using low p53 producer cells to screen a variety of cancer therapy drugs which may cooperate with p53 to induce apoptosis. Thus, it is hoped that more effective chemotherapeutic drugs can eventually be identified.

FUTURE WORK

With collaboration of Philip Friedlander, a graduate student at Carol Prives laboratory, we tried several times to develop the breast cell lines expressing inducible p53 during last two years. As detailed in the section of Materials and Methods, we used a two step procedure. While we were successful in making saos-2 and H1299 cells expressing inducible p53, we failed every time so far to generate the inducible breast cells. We used two p53-null breast cell lines, MB-MDA-157 and MB-MDA-453. We found MDA-MB-157 is extremely sensitive to the harsh condition imposed by the transient transfection assay, presumably due to the CaCl. As for MDA-MB-453, we got more than 20 clonal cell lines that were transfected with pUHD15-1 neo after several experiments. Of those cell lines four (M5, M9, M11 and M14) were found to express p53 in the absence of tetracycline by transient transfection assay. Next, 10-3 plasmid containing cDNA encoding wild type p53 was cotransfected with the puromycin selectable pBabe plasmid (Morgenstern and Land 1990) into either M5, M9, M11 or M14. Clones were selected and maintained in the presence of 2 μg and 1 μg of puromycin (Sigma) per ml, respectively. More than 50 single clonal cell lines were selected and only twelve of those were grown up. Individual clones were then screened for inducible expression of the p53 protein by Western blot analysis using monoclonal antibodies against p53. None of those clonal cell lines expressed p53. This may be due to basal or leaky expression from the promoter of 10-3 plasmid in MDA-MB-453. Thus the breast cell line MDA-MB-453 is probably extremely sensitive to very low level of wild-type p53 expression. In addition, the doubling time for MDA-MB-453 cells in our hand is about 5-7 days, which is 2 and 3 times longer than saos-2 and H1299 cells, respectively. Taken together, this is why I am still in the process of developing breast cell lines expressing p53 under tetracycline regulated promoter.

To circumvent the problem, we'll use electroporator to transfect MDA-MB-157 and MDA-MB-453 cells with 15-1 neo and get more clonal cell lines and try the whole process all over again. We hope we can generate inducible breast cell lines in about 6 months. Currently it is in progress.

Once these cell lines are generated, we will determine the cooperativity of p53 with cyclin D1 in inducing apoptosis. If we can not generate stable cell lines expressing inducible p53, we'll determine the cooperativity of p53 with cyclin D1 in inducing apoptosis by transient transfection assay.

Furthermore, we will determine the domain of p53 that cooperates with cyclin D1 in inducing apoptosis by transient transfection assay.

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APPENDIX A.

MATERIALS AND METHODS

Plasmids. The pUHD15-1 neo plasmid contains the tTA transactivator gene derived from pUHD15-1 and the neomycin gene as described in Resnitzky et al. (1994). Genes of interest were cloned into the 10-3 plasmid to allow their conditional expression as described in Gossen and Bujard, (1992). The pBabe plasmid contains the puromycin resistance gene (Morgenstein and Land, 1990) and was used for secondary selection as described below. To construct tetracycline regulated expression vectors, the following cDNA fragments were cloned into the 10-3 vector: wild-type p53 (Baker er al. 1990), tumor-derived mutant forms of p53 (his 175 and ser 249) (Baker et al. 1990), the transactivation-deficient form of p53 (gln22/ser23) (Lin et al., 1993), deletion mutants lacking either N-terminal 22 (ΔN22), 96 (ΔN96) or C-terminal 30 (ΔC30) amino acids (Jayaraman and Prives, unpublished), a transactivation defective form of p53 which lacks the C-terminal 30 amino acids (gln22/ser23ΔC30) (see below for construct generation), or p21 (WAF1) (El-Deiry et al. 1993). To generate p53 (gln22/ser23ΔC30), the C-terminus of the p53 (gln22/ser23) cDNA beginning at amino acid 144 at the PvuII site was replaced by the C-terminus of the ΔC30 p53 cDNA.

Cell lines and transfection, and selection procedures. The Saos-2 and H1299 cells were purchased from the American Type Culture Collection. RKO cells were obtained from M. B. Kastan (Kastan et al. 1992). All cells were grown in Dulbocco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum in a 37oC incubator with 5% CO2. Transfections were performed using the calcium chloride method as described (Chen and Okayama, 1987). To generate cell lines expressing inducible proteins of interest, a two step procedure described by Resnitzky et al. (1994) was used as summarized below. First, low passage Saos-2 or H1299 cells were transfected with pUHD15-1 neo and clones were selected and maintained in the presence of 400 and 250 μg/ml of active G418 (Geneticin, Gibco), respectively. To test for cloned cell lines capable of inducing expression from the tetracycline promoter, the 10 3(p53) plasmid was transiently transfected into those cells in the presence of tetracycline (1 μg/ml) for 10-18 h and the transfected cells were then split 1:2 and grown in the presence or absence of tetracycline. Cells were extracted 24-48 h later, and expression of the p53 protein was determined by Western blot analysis. Two of the 15 clonal Saos-2 cell lines (S32 and S2) and two of the 30 clonal H1299 cell lines (H15 and H24) were found to induce p53 expression upon withdrawl of tetracycline. Since p53 is a very potent suppressor of cell growth, cell lines with "leaky" expression of p53 could not be stably propagated. To ascertain that these cells do not induce expression from the tetracycline promoter in the presence of tetracycline, they were cotransfected by 10-3(p53) and a plasmid containing a luciferase reporter gene under control of the gadd45 p53 responsive element (gadd45-luc) (Chen et al. 1995). The S32 and H24 cell lines were found to induce lower levels of basal luciferase activity in the presence of tetracycline than S2 and H24 cell lines, respectively. In addition, the fold of induction of luciferase activity in the absence of tetracycline is two times higher in S32 and H24 cells than in S2 and H15 cells, respectively. Therefore, both S32 and H24 cell lines were used as parental cell lines for subsequent generation of inducible cell lines. Second, various 10-3 plasmids containing cDNAs encoding either wild type, or mutant forms of p53 or p21 were cotransfected with the puromycin selectable pBabe plasmid (Morgenstern and Land 1990) into either S32 or H24 cells. Clones were selected and maintained in the presence of 2 μg and 1 μg of puromycin (Sigma) per ml, respectively. Individual clones were screened for inducible expression of the p53 and p21 proteins by Western blot analysis using monoclonal antibodies against p53 and p21 as described below.

Immunoblot analysis. Cells were collected from plates in phosphate-buffered saline (PBS) and resuspended with 1x sample buffer, and boiled for 5 min. For immunoblot analysis, a standard procedure was followed as previously described (Chen et al. 1995). The various

monoclonal antibodies used to detect p53 were PAb1801 and PAb421 as described (Chen et al., 1995). The affinity-purified monoclonal antibody against p21 (Ab-1), Bcl-2 (Ab-1) was purchased from Oncogene Science (Uniondale, NY) and affinity-purified monoclonal antibodies against Bax (P-19) and anti-actin polyclonal antibodies was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Sigma (St. Louis, MO), respectively.

Growth rate and cell cycle analyses. To determine the rate of cell growth, 1 x 10^5 cells were seeded per 60-mm plate with or without tetracycline. The medium was replaced with fresh medium with or without tetracycline every 48h. At indicated times, cells were trypsinized and collected individually from at least two plates. Cells from each plate were counted with a hemacytometer at least twice. The average number of cells from at least two plates were used for growth rate determination.

For cell cycle analysis, 2.5 x 10^5 cells were seeded per 90-mm plate with or without tetracycline. The medium was replaced every 48 h as needed with fresh medium with or without tetracycline. At the indicated times, cell were trypsinized and fixed with 2 ml of 70 % ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 µg/ml each of RNase A (Sigma) and propidium iodide (PI) (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 hours. The percentage of cells in various cell cycle phases was determined by using the CellFit program.

APPENDIX B. TABLE 1

Protein expressed [a]		Saos-2 [b]	H1299 [b]	Apoptosis [c]	Arrest [c]	Cycling [c]
Wild-type p53 (H)	1 — 393	1	2	+++	+ [d]	-
Wild-type p53 (L)		1	5	-	+	-
p53 (ΔN22) (H)	23 — 393	nd	2	+++	+ [d]	-
p53 (ΔN22) (L)		nd	2	-	+	-
p53 (22/23) (H)	1 — gln22/ser23 — 393	3	2	+	-	-
p53 (22/23) (L)		3	3	-	-	+
p53 (ΔC30) (H)	1 — 363	2	2	+	+	-
p53 (ΔC30) (L)		3	2	-	+	-
p53 (22/23 ΔC30) (H)	1 — gln22/ser23 — 363	nd	1	-	-	+
p53 (ΔN96) (H)	97 — 393	4	1	-	-	+
p53 (ser249) (H)	1 — ser249 — 393	4	4	-	-	+
p53 (his175) (H)	1 — his175 — 393	4	3	-	-	+
p21 (H)	1 — 164	3	3	-	+	-

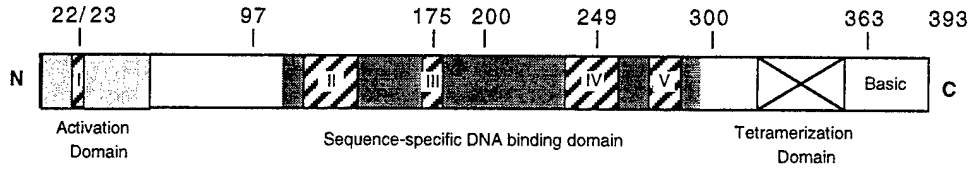


Table 1. Cell lines expressing inducible p53 or p21.

(a) Proteins were detected by Western blotting with p53 specific monoclonal antibodies PAb1801 or PAb421, or p21 specific monoclonal antibody AB-1 (Oncogene Science). Clones were divided where possible into high (H) and low (L) producers when protein levels differed by at least two-fold.
(b) Number of individual clones of Saos-2 or H1299 cells expressing inducible p53 or p21.
(c) Apoptosis , arrest or cycling states of cells were determined by growth curves and FACS analysis and results obtained were essentially similar for Saos-2 and H1299 cells.
(d) In cells which underwent massive apoptosis, cell arrest was transient.

APPENDIX C.

FIGURE LEGENDS

Fig. 1. Induction of apoptosis by high levels of p53 in p53-7 cells. (A) Inducible expression of p53 and levels of actin in p53-7 cells in the absence or presence of tetracycline (1 μg/ml) for 24 h, and in RKO cells treated with or without camptothecin (200 nM/ml) were assayed by western blot analysis. The blot was probed with p53 monoclonal PAb1801 and actin polyclonal antibodies. (B) Growth rates of p53-7 cells in the presence or absence of tetracycline were measured as described in Materials and Methods (Appendix A). (C) DNA contents were quantitated by propidium iodide staining of fixed cells at 0, 1, 2 and 3 days following withdrawal of tetracycline as described in Materials and Methods (Appendix A). Bottom panel. The percentage of cells in the Go-G1, S, G2-M, and sub-G1 phases of cell cycle at 0, 1, 2, and 3 days following withdrawal of tetracycline was calculated from flow cytometric measurements of DNA content.

Fig. 2. Induction of cell cycle arrest by low levels of p53 in p53-13 cells. The experiments were performed in an identical manner to those in Fig. 1.

Fig. 3. The level of p53 determines cell death or arrest. (A) Inducible expression of p53, and levels of actin in p53-7 cells in the presence of 1,000, 40, 20, 10, 8, 6, 4, 2, 1, and 0 ng/ml of tetracycline as indicated were assayed by western blot analysis. The blots were probed with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies. (B) The growth rates of p53-7 cells in the presence of varying concentrations of tetracycline were measured as described in Materials and Methods (Appendix A).

Fig. 4. p53 sensitizes cells to undergo apoptosis by CPT-induced DNA damage. (A) Levels of p53, p21 and actin were assayed by western blot analysis in p53-13 cells in the presence or absence of tetracycline for 24 h, followed by treatment with CPT at concentrations of 0, 50, 100, 200, and 400 nM/ml for another 24 h. (B) Number of apoptotic cells of the untreated or CPT-treated p53-13 cells in the presence or absence of tetracycline.

Fig. 5. p21 induction lead to cell cycle arrest but not apoptosis. The experiments were performed as those in Fig. 1.

Fig. 6. Induction of a delayed apoptosis but not arrest by p53 transactivation defective mutant (gln22/ser23). DNA contents were quantitated by flow cytometric analysis in 22/23-4 cells at day 0, 3 and 6 following withdrawal of tetracycline. The percentage of cells in various phases of cell cycle at day 0, 3, and 6 after withdrawal of tetracycline.

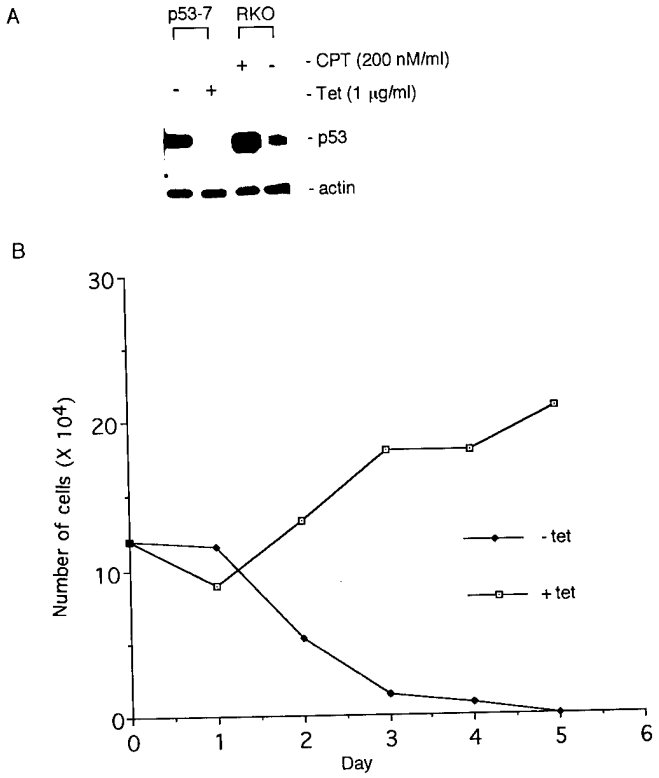
Fig. 7. The C-terminus of p53 is required for full apoptosis. (A) Inducible expression of wild-type p53 and p53(ΔC30), and levels of actin in p53-7, ΔC30-9, ΔC30 6 and ΔC30-12 cell lines in the presence or absence of tetracycline (1 μg/ml) for 24 h. The blot was reacted with p53 monoclonal PAb1801 and actin polyclonal antibodies. (B) The inducible expression of wild-type p53, p53 (ΔC30) and p21, and levels of actin in p53-7 and ΔC30-6 cells in the presence or absence of tetracycline (1 μg/ml) for 24 h. The blot was reacted with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies, and p21 monoclonal antibody, respectively. (C) and (D) The experiments were performed in an identical manner to those in FIg. 1B and 1C. See Fig. 1 legend.

Fig. 8. A full apoptotic response requires intact wild-type p53. Summary of the percentage of cells with sub-G1 DNA contents which express inducible wild-type p53, p53 (ΔC30), p53 (gln22/ser23), p53 (ΔN96), p53 (his175) and p53 (ser249) at various times following withdrawal of tetracycline.

Fig. 9. Levels of bax and bcl-2 are not affected by strong induction of p53 leading to apoptosis in Saos-2 cells. The levels of bax and bcl-2 proteins in p53-7 cells in the presence or absence of tetracycline were quantitated by western blot analysis. The four blots shown were probed with a mixture of p53 monoclonal PAb1801 and actin polyclonal, p21 monoclonal, bax polyclonal, and bcl-2 monoclonal antibodies, respectively.

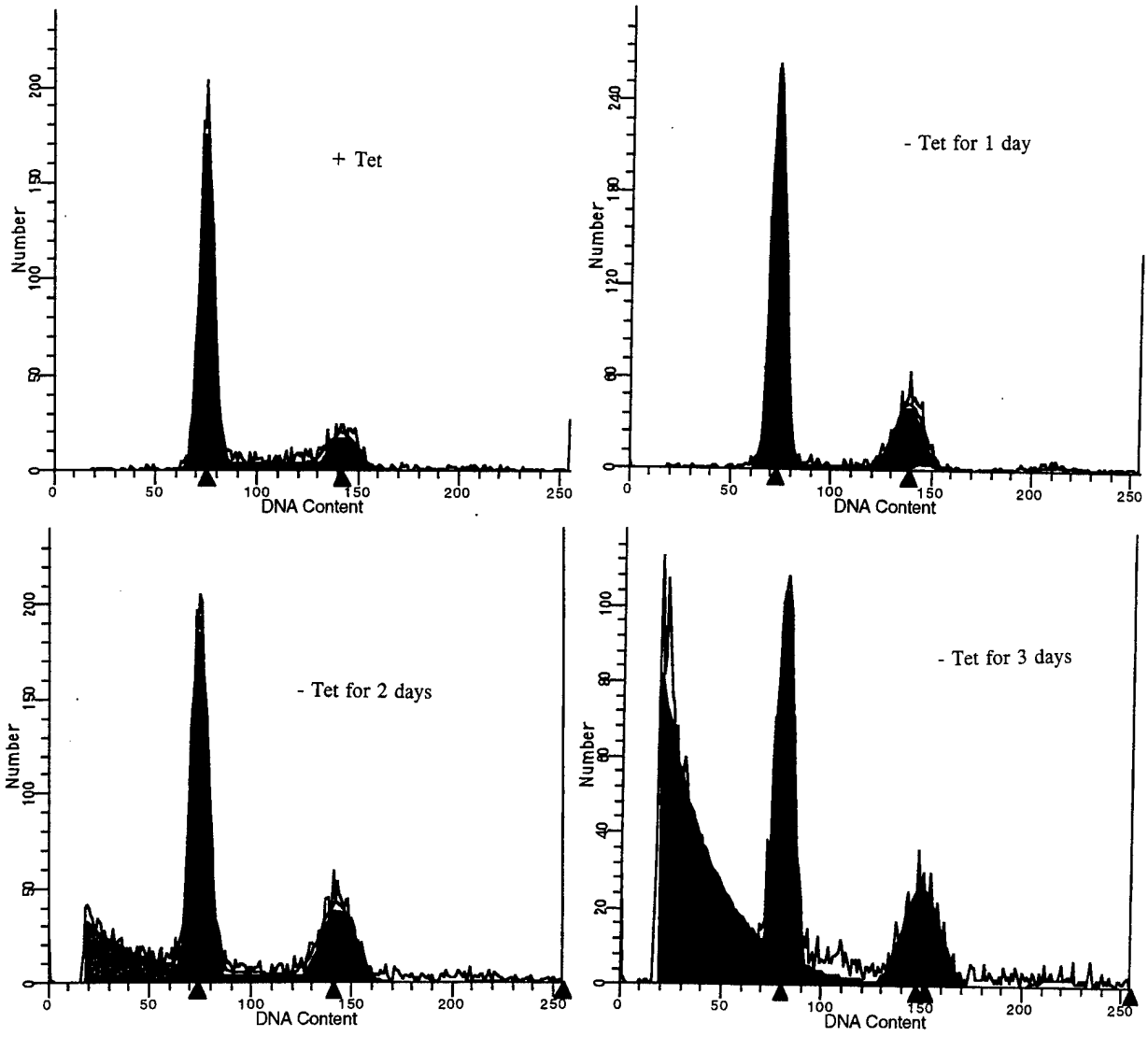
Fig. 10. A model of p53-dependent apoptosis.

APPENDIX D. Fig. 1



APPENDIX D. Fig. 1

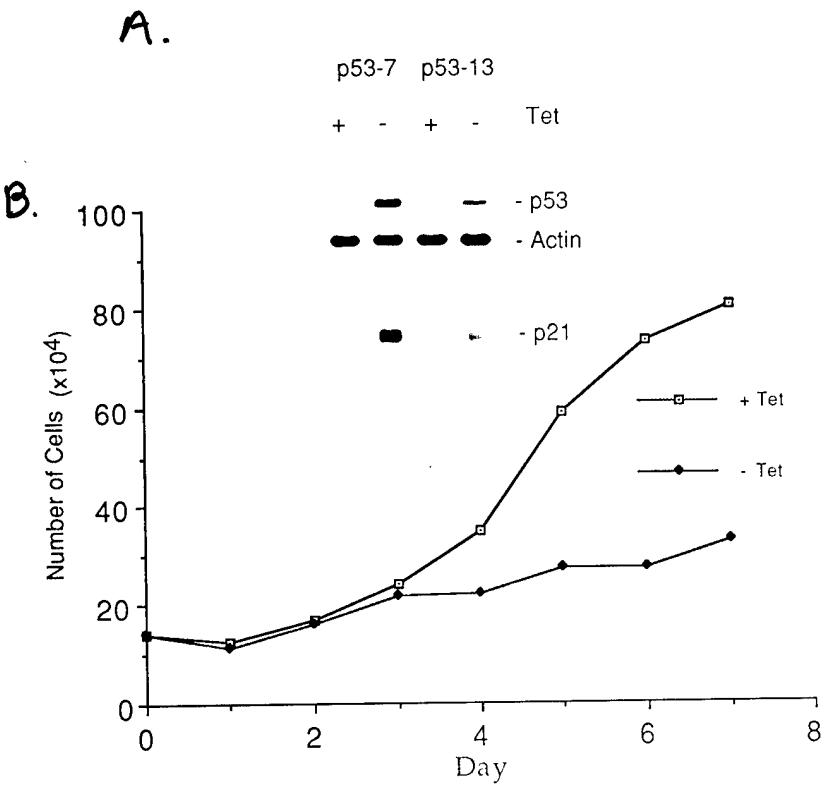
C



p53-7 cell line Time of p53 expression (D)

	0 D	1 D	2 D	3 D
G_0-G_1	71.6	72.38	47.85	36.11
S	14.03	4.94	7.28	0
G_2-M	14.37	22.68	19.94	3.46
Sub-G_1	0	0	24.92	60.43

APPENDIX D. Fig. 2



APPENDIX D. Fig. 2

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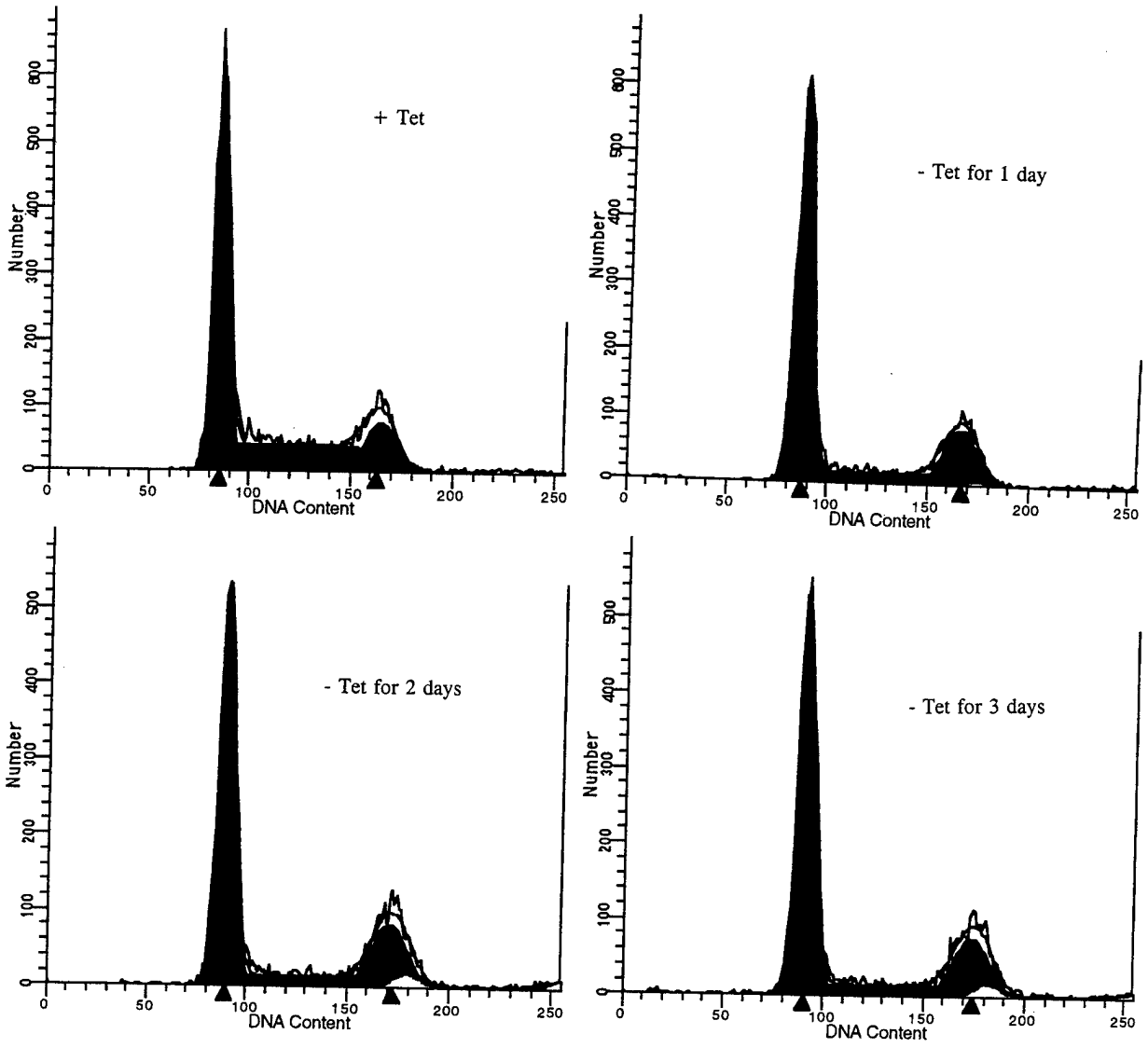
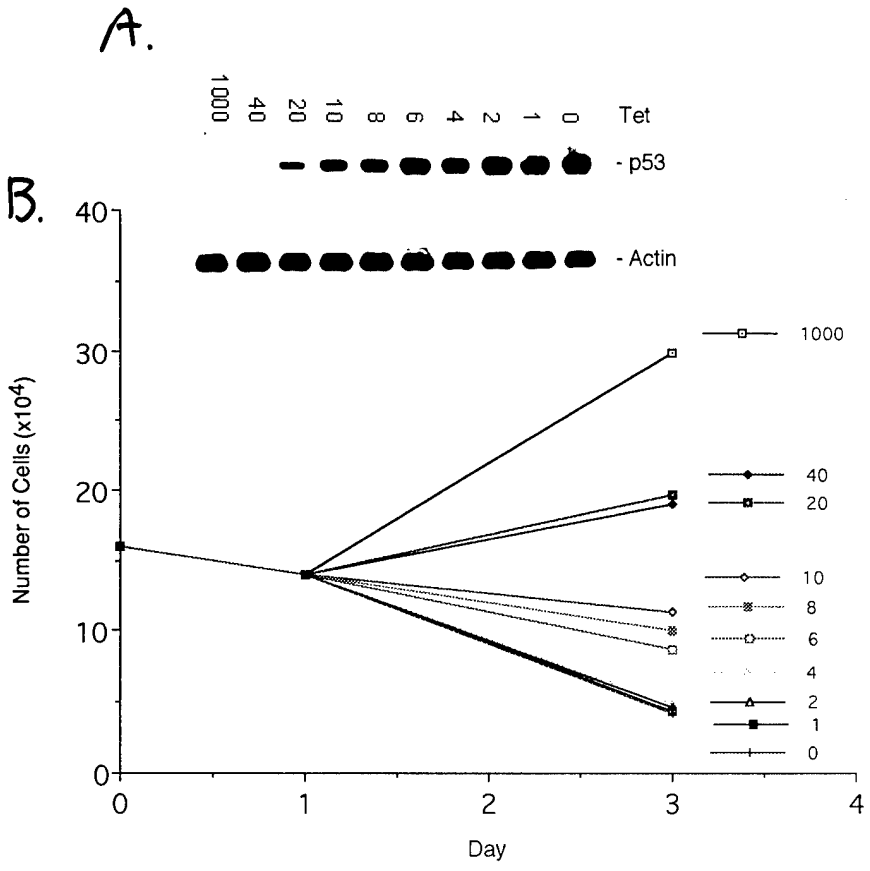


p53-13 cell line Time of p53 expression (D)

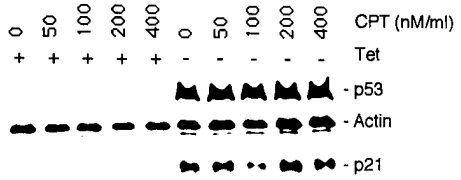
	0 D	1 D	2 D	3 D
G_0-G_1	54.99	66.71	65.12	65.64
S	31.12	14.70	14.12	14.95
G_2-M	13.89	18.59	20.77	19.41
Sub-G_1	0	0	0	0

APPENDIX D. Fig. 3



APPENDIX D. Fig. 4

A



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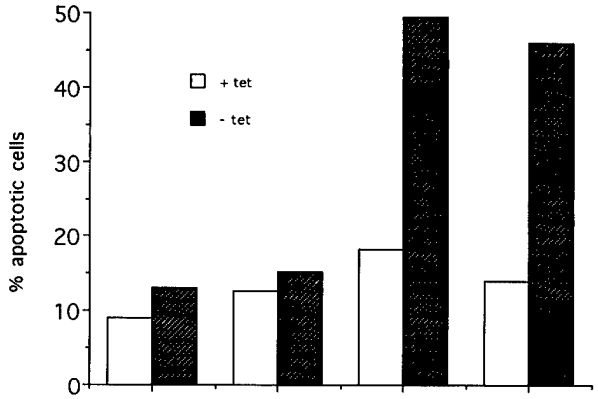
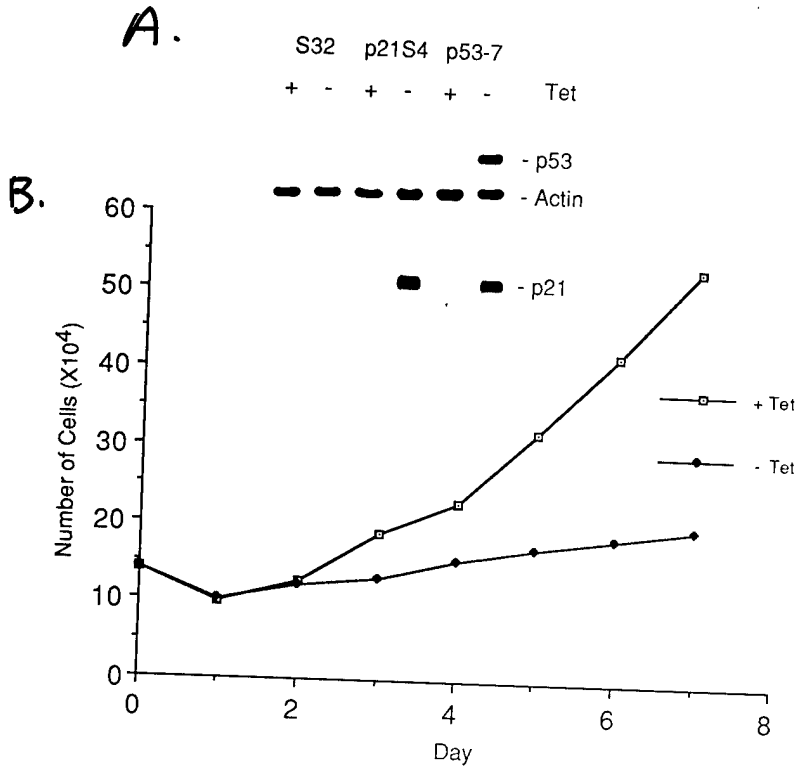


CPT (nM/ml)	50	100	200	400
Fold increase with p53	1.44	1.21	3.12	3.5

APPENDIX D. Fig. 5



APPENDIX D. Fig. 5

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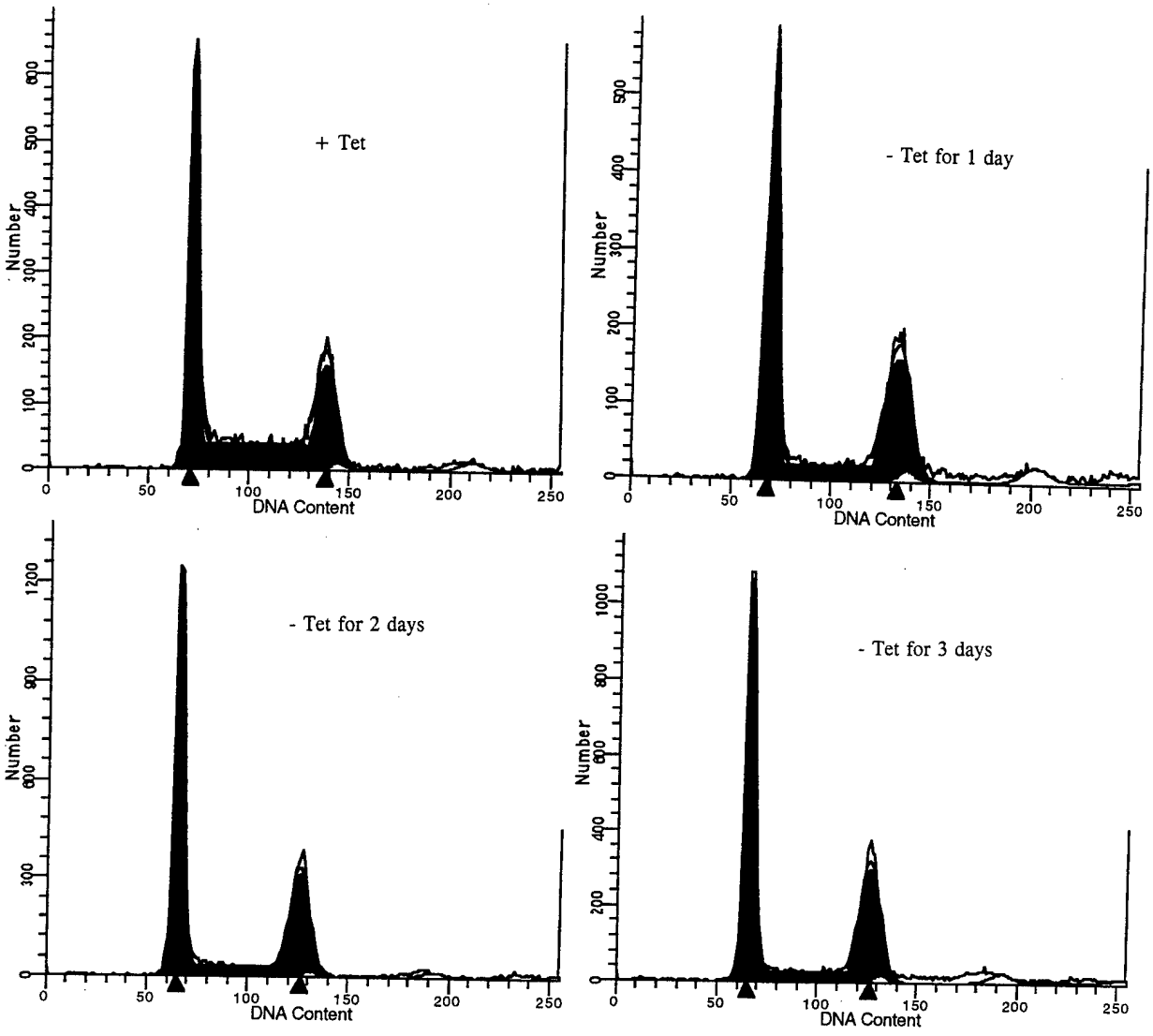
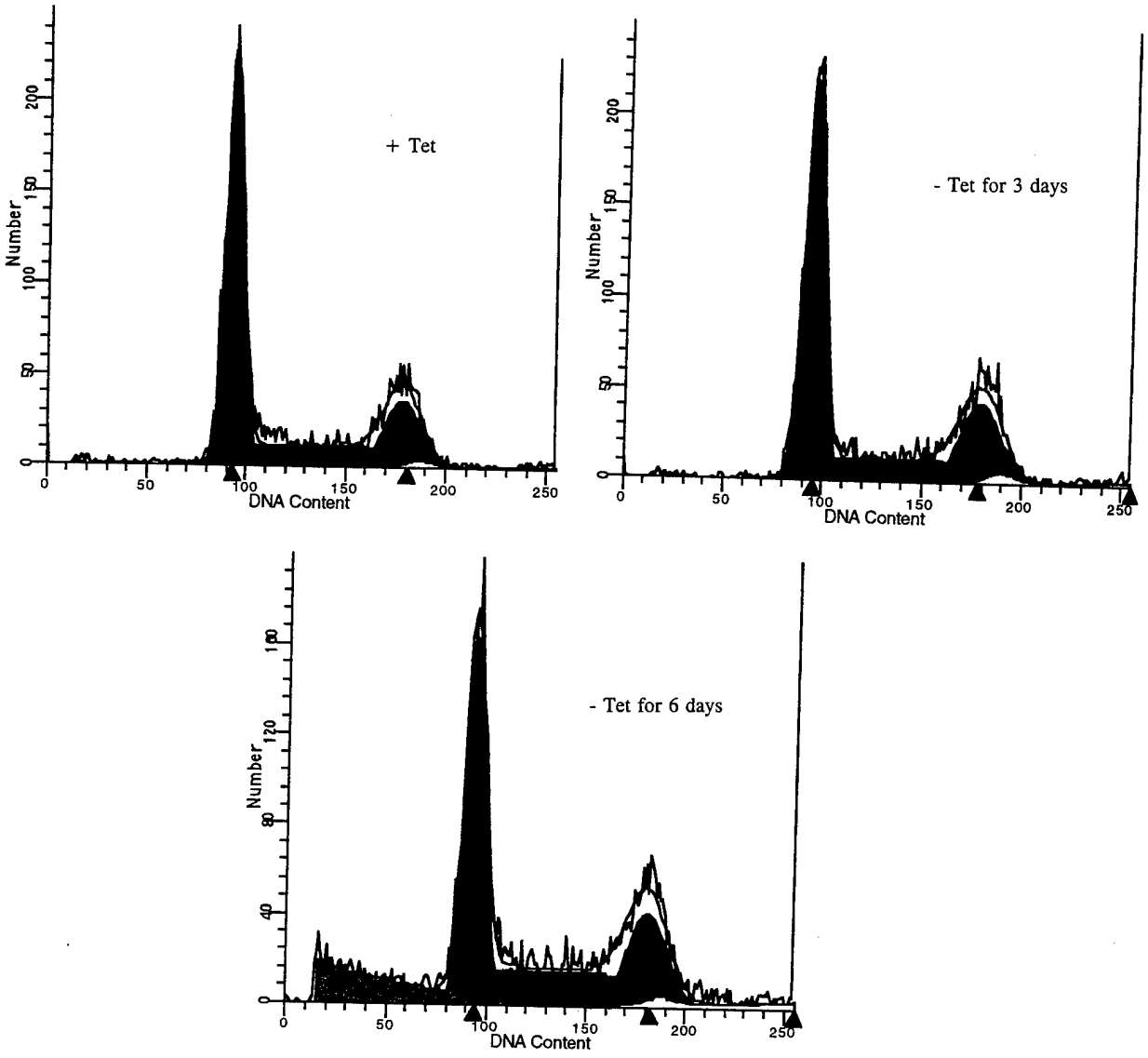


p21S4 cell line Time of p21expression (D)

	0 D	1 D	2 D	3 D
G_0-G_1	44.7	51.56	57.14	55.27
S	32.29	17.0	14.49	14.0
G_2-M	23.0	31.44	28.37	30.72
Sub-G_1	0	0	0	0

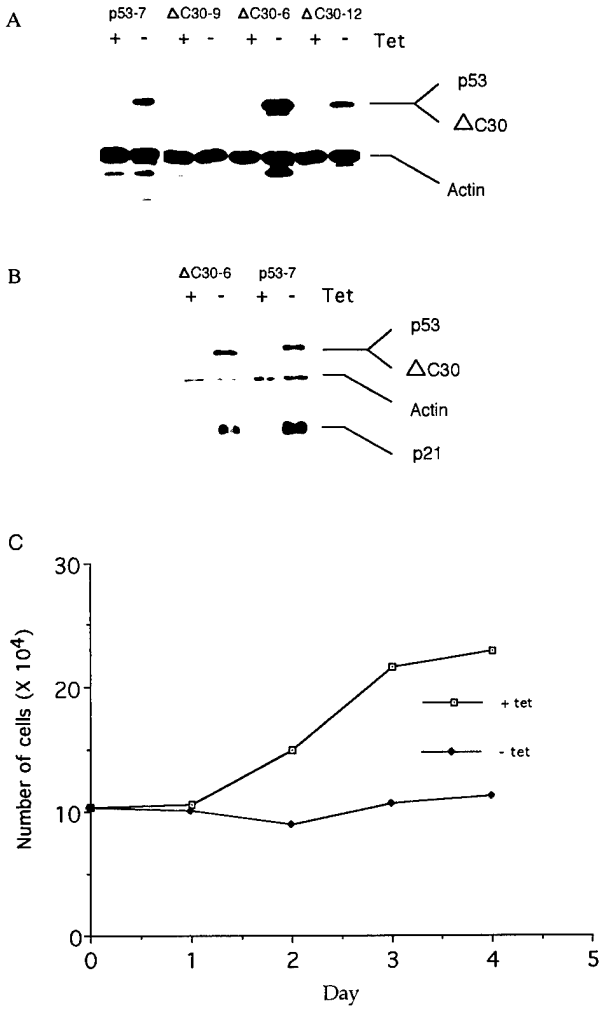
Fig. 6



22/23-4 cell line Time of p53 expression (D)

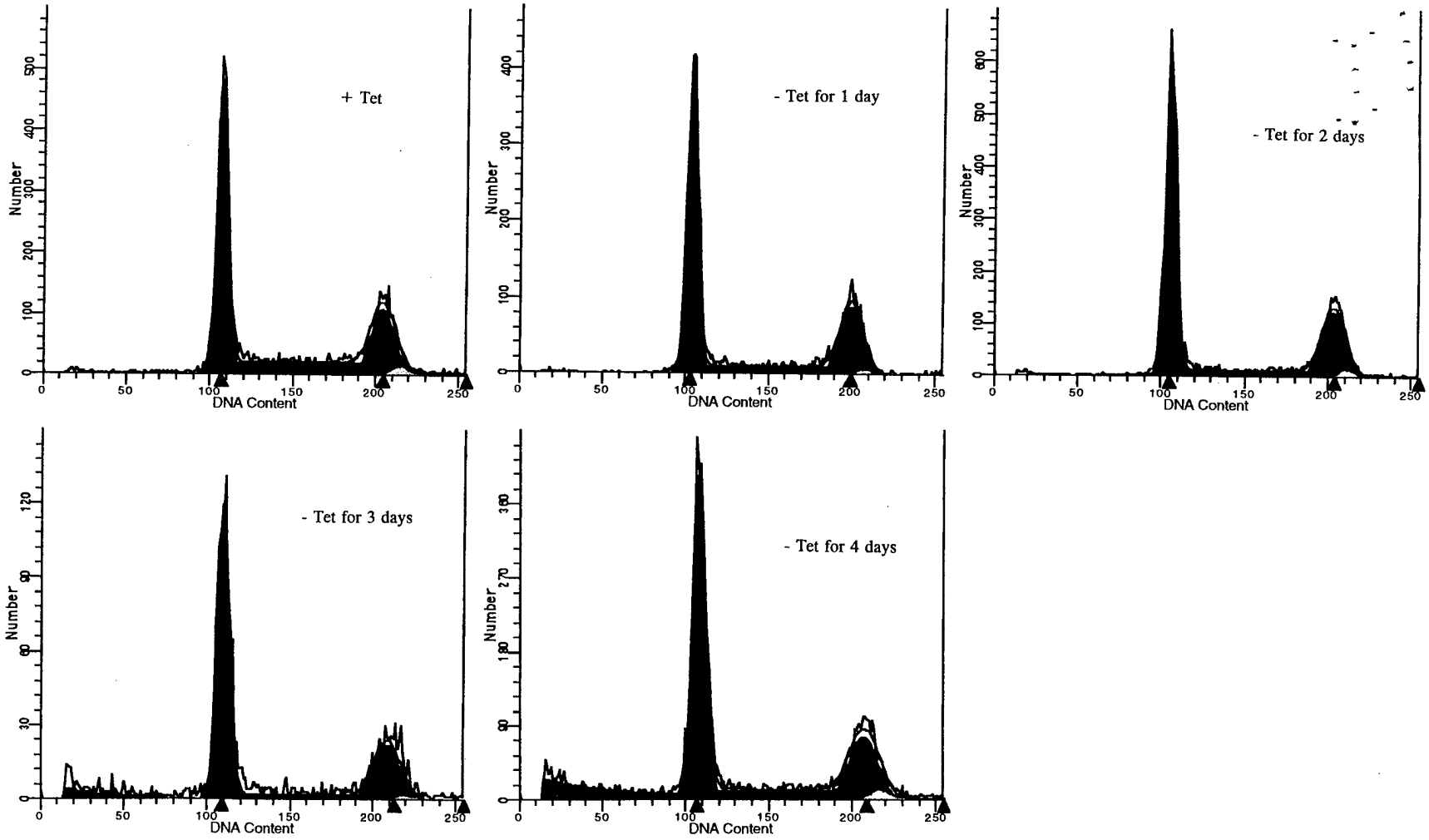
	0 D	3 D	6 D
G_0-G_1	56.94	55.02	38.49
S	23.67	22.69	24.73
G_2-M	19.39	22.29	19.71
Sub-G_1	0	0	17.06

APPENDIX D. Fig. 7



APPENDIX D. Fig. 7

D.

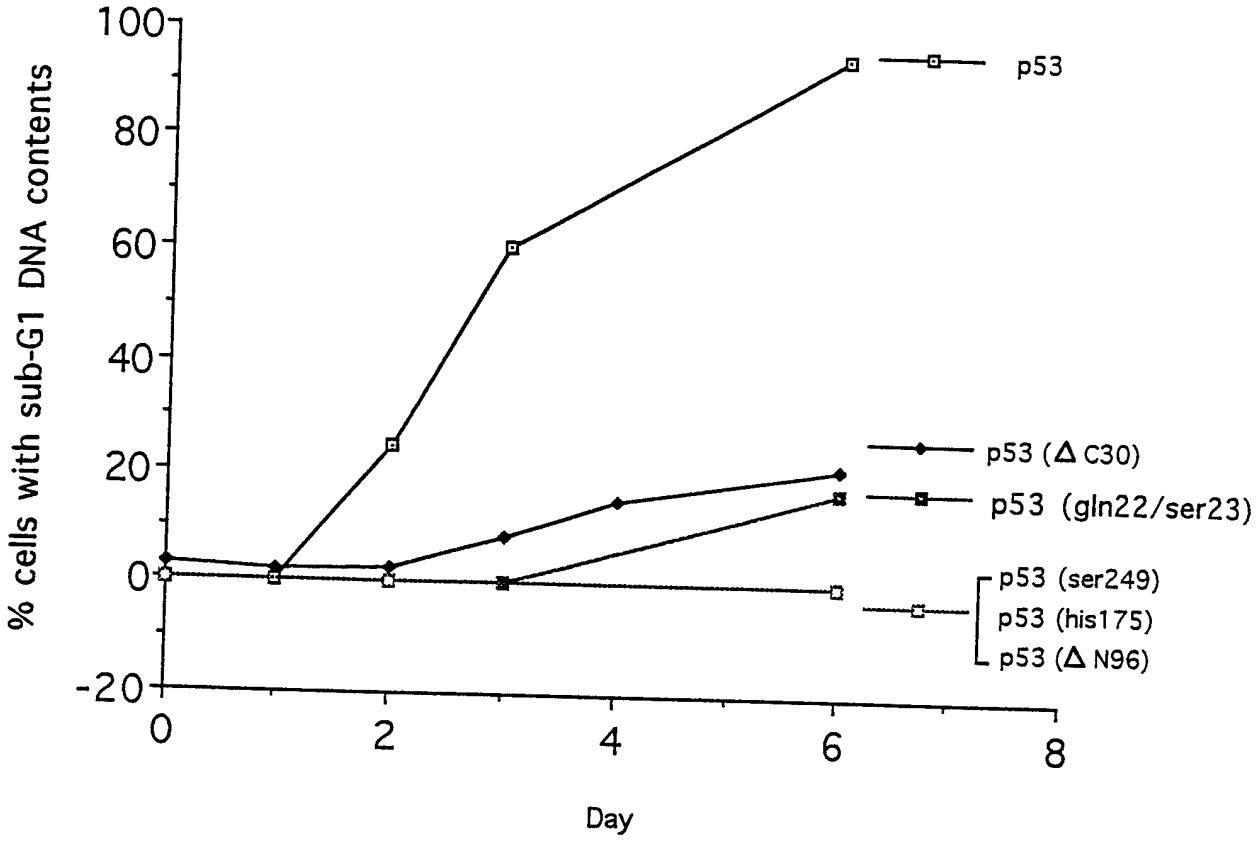


△ C30-6 cell line

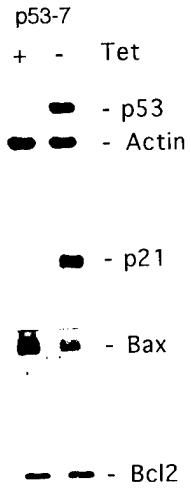
Time of p53 expression (D)

	0 D	1 D	2 D	3 D	4 D
G_0-G_1	49.76	57.12	59.64	58.33	50.34
S	24.41	16.13	13.57	10.86	14.48
G_2-M	22.85	24.80	24.28	22.29	20.15
Sub-G_1	2.98	1.95	2.51	8.52	15.04

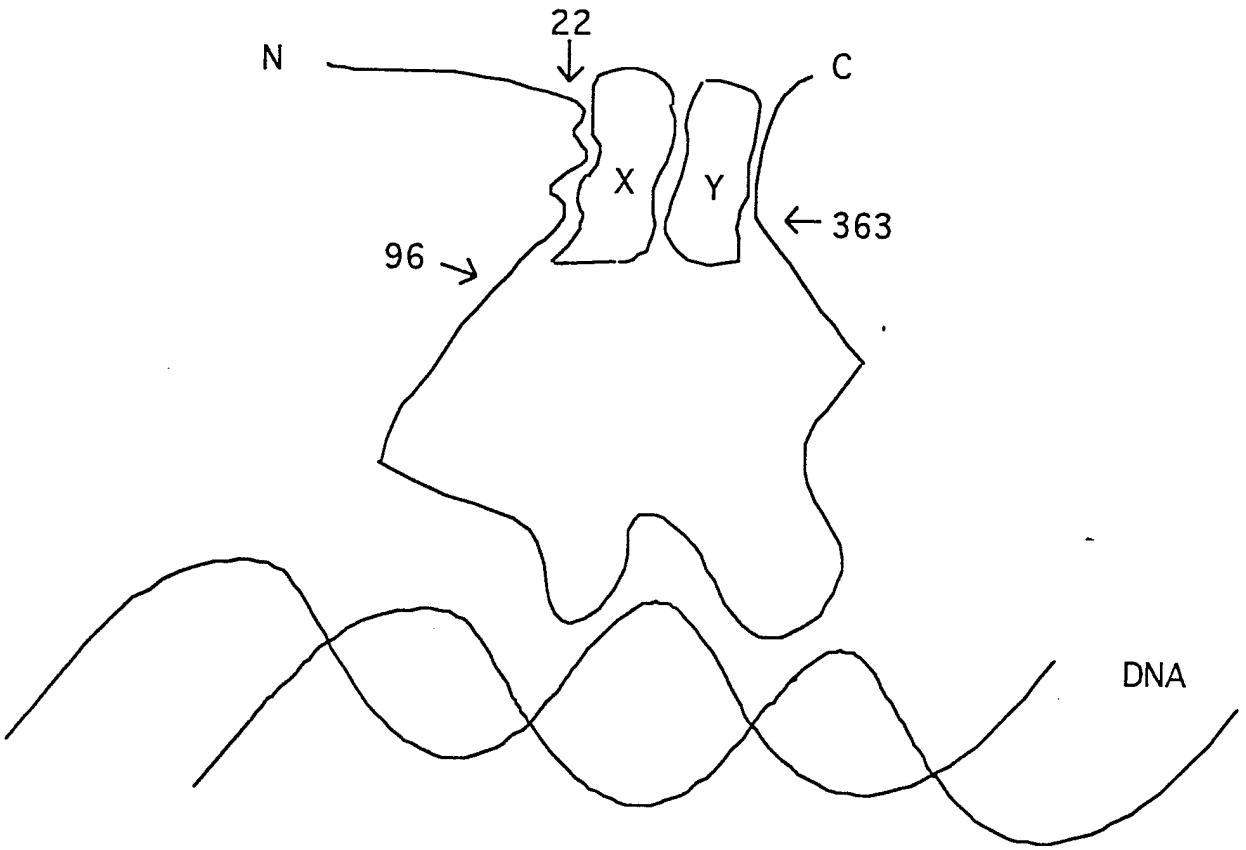
APPENDIX D. Fig. 8



APPENDIX D. Fig. 9



APPENDIX D. Fig. 10



[CANCER RESEARCH 55, 4257–4263, October 1, 1995]

Advances in Brief

p53, through p21 (WAF1/CIP1), Induces Cyclin D1 Synthesis[1]

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Abstract

Cells induced to accumulate the p53 tumor suppressor protein have been shown to arrest in G1. This arrest is characterized by accumulation of the cyclin-dependent kinase inhibitor p21 (WAF1/CIP1) and of underphosphorylated forms of retinoblastoma protein. We show here that accumulation of the wild-type p53 protein in either human or murine cells markedly increases expression of cyclin D1. The induction of cyclin D1 can also be mediated by a target of p53, the p21 (WAF1/CIP1) inhibitor of cyclin-dependent kinases. The relationship between the induction of cyclin D1 and G_1 arrest defines a new cellular response to p53.

Introduction

In response to both intrinsic and environmental DNA damage agents, the steady-state level of the p53 protein is posttranscriptionally elevated (1, 2). It is well established that accumulation of the wild-type p53 protein results in two pathways, cell cycle arrest and programmed cell death or apoptosis, which together carry out the p53 tumor suppressor function (3, 4). What pathway the DNA damaged cells undergo depends on both the extracellular signals, the threshold of different cell types for apoptotic inducers, and expression of other cellular and viral proteins (5). Mutation of p53, which may be the most common event in human cancers, leads to disruption of these pathways, resulting in a selective growth advantage of tumor cells, generally observed as tumor progression.

It is now fairly well established that p53-induced cell growth arrest is due to the ability of p53 as a transcriptional activator to regulate one or more cell cycle checkpoint-related genes. Among the genes that were shown to be induced by p53 in cells are *mdm-2* (6), *GADD45* (2), and *p21* (WAF1/CIP1) (7). Of these three, the *p21* gene is the most likely to directly regulate the cell cycle. It has been shown that its product was found to be a potent CDK[3] inhibitor (8, 9), as well as to inhibit DNA replication (10) but not PCNA-dependent DNA repair (11) through its physical interaction with PCNA. Therefore, p21 can disrupt the normal progression of DNA-damaged eukaryotic cells through the cell cycle. These findings provide a direct link between p53 tumor suppressor protein and cell cycle control.

Accumulation of the wild-type p53 protein primarily arrests cells at G_1-S (3, 4). Cell cycle transition from G_1 to S phases requires sequential events involving the formation, activation, and subsequent inactivation of a series of cyclins/CDK complexes. A critical target of G1 CDKs is the pRB protein whose normal function of repressing transcription factors, including members of the E2F family, is inhibited by CDK phosphorylation (12). Inhibition of CDK4 synthesis by transforming growth factor β1 is linked to cell cycle arrest (13). Additionally, regulation of the synthesis and activity of the other cyclins and the activity of cyclin/CDK complexes has been shown to play a major role in cell cycle control (14, 15).

As part of an effort to identify important cell cycle-related genes that are potentially regulated by p53, we have found that cyclin D1 expression is induced by the wild-type p53 protein. This induction is at least partially mediated by p21.

Materials and Methods

Cells, Antibodies, and Plasmid Construction. Saos-2, WI-38, and T98G cells were purchased from the American Type Culture Collection. RKO cells were obtained from M. B. Kastan (The Johns Hopkins University, Baltimore, MD; Ref. 2). 10(1) cells were obtained from A. J. Levine (Princeton University, Princeton, NJ; Ref. 16). 3-4 cells were generated by cotransfection of 10(1) cells with the activated H-*ras* oncogene and the temperature-sensitive murine $p53^{val135}$. GM47–23 and Del4A cells were generously provided by W. E. Mercer (Thomas Jefferson University, Philadelphia, PA; Ref. 17). All cells were grown in DMEM supplemented with 10% fetal bovine serum in a 37°C incubator with 5% CO_2. Transfection was performed by the calcium phosphate method as described (18). Camptothecin was purchased from Sigma Chemical Co. (St. Louis, MO). Affinity-purified mAbs against PCNA and cyclins B1 and D1 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Affinity-purified polyclonal antibody against cyclin D1 was purchased from Upstate Biotechnology, Inc. (cyclin D1; UBI, Lake Placid, NY). Rabbit polyclonal antisera against CDK4, CDK2, and cdc2 were kindly provided by Z-Q. Pan (Mount Sinai Medical Center, New York, NY; Ref. 19). Anti-cyclin A (C160; Ref. 20) and anti-pRB (XZ-77; Ref. 21) were mouse mAbs. Affinity-purified anti-actin antibody was purchased from Sigma. PAb 419 is a mAb against SV40 large T antigen (22). PAb1801 and PAb122 are mAbs against p53 (23, 24). To generate anti-p21 antibody, a 900-bp *StuI-EcoRI* cDNA fragment encoding amino acids 17 to 164 of p21 polypeptide (7) was inserted in-frame into pRSET expression vector (Invitrogen). The His-tagged p21 protein was then produced in bacteria and purified from Ni-agarose beads, and anti-p21 antibody was raised in a rabbit. pcDNA3-p21 was generated by inserting a 1.0-kb *EcoRI-EcoRI* fragment of p21 cDNA (7) into pcDNA3 (Invitrogen).

Immunoprecipitation and Immunoblot Analysis. For immunoprecipitation analysis, cells were grown in DMEM (methionine minus) media plus 10% fetal bovine serum for 1 h and labeled with 75 μCi/ml of Tran^{35}S-label methionine (ICN Pharmaceuticals) for 1 h. Whole-cell extracts were prepared by lysing cells with NP40 lysis buffer [150 mM NaCl, 50 mM Tris (pH 7.5), 1 mM DTT, 0.5% NP40, 25 ng/ml aprotinin, 25 ng/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] for 15 min on ice. The extracts were precleared with 10 μl of normal rabbit serum plus 100 μl of 10% *Staphylococcus aureus* cells (The Enzyme Center, Malden, MA) for 1 h at 4°C and were clarified by centrifugation. Immunoprecipitations were performed by the addition of various antibodies as indicated and 40 μl of 50% protein A-Sepharose (Pharmacia). The immunoprecipitates were washed three times with NP40 lysis buffer prior to the addition of 2× sample buffer and heat at 100°C for 5 min. Polypeptides were resolved by SDS-PAGE and fluorography.

For immunoblot analysis, cells were lysed with the NP40 lysis buffer, then mixed with 2× sample buffer, and boiled for 5 min. Following electrophoresis, proteins were transferred to a nitrocellulose filter. Blots were blocked by incubation with PBS containing 0.1% Tween 20 (PBS-T) and 2% nonfat milk for 30 min at room temperature. The filter was incubated with antibody as indicated in PBS-T, then washed three times with PBS-T, and incubated with a 1:2000 dilution of either anti-mouse or anti-rabbit immunoglobulin G conjugated to horseradish peroxidase (Organon Teknika Corp., Durham, NC) for

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[2] To whom requests for reprints should be addressed.

[3] The abbreviations used are: CDK, cyclin-dependent kinase; PCNA, proliferating cell nuclear antigen; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

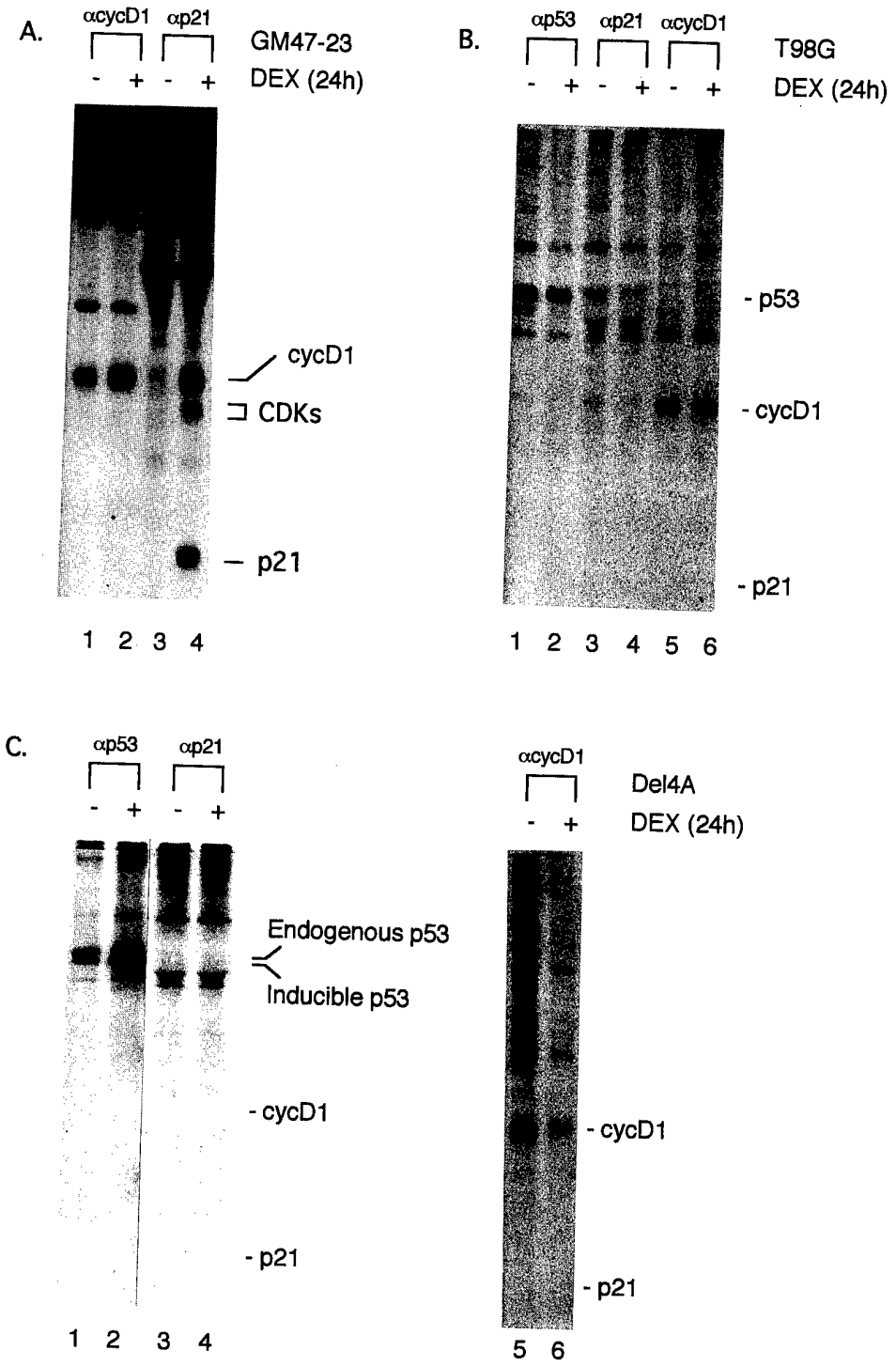

Fig. 2. p53-dependent induction of cyclin D1 synthesis. *A*, GM47–23 cells treated with dexamethasone for 0 or 24 h were [^{35}S]methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against cyclin D1 (*Lanes 1* and *2*) and p21 (*Lanes 3* and *4*). T98G cells (*B*) or Del4A cells (*C*) treated with dexamethasone for 0 or 24 h were [^{35}S]methionine labeled. The labeled cell extracts were subject to immunoprecipitation with antibodies against p53 (PAb1801; *Lanes 1* and *2*), p21 (*Lanes 3* and *4*), and cyclin D1 (*Lanes 5* and *6*). *Right*, cyclin D1, p21, and inducible and endogenous mutant p53 proteins.

p53-dependent Induction of Cyclin D1 Is at the Transcriptional Level. To determine whether accumulation of the wild-type p53 protein stabilizes the cyclin D1 protein, the half-life of the cyclin D1 protein in GM47–23 cells and the normal human cell line WI-38 was determined. From the results obtained, it was clear that the cyclin D1 protein has the same half-life (approximately 15–30 min) both in GM47–23 cells, either in the presence or in the absence of the wild-type p53 protein, and also in another human cell line (WI-38 cells; data not shown). Thus, induction of cyclin D1 by p53 is not due to protein stabilization.

To analyze whether induction of cyclin D1 expression is at the transcriptional level, cyclin D1 mRNA was quantitated by Northern

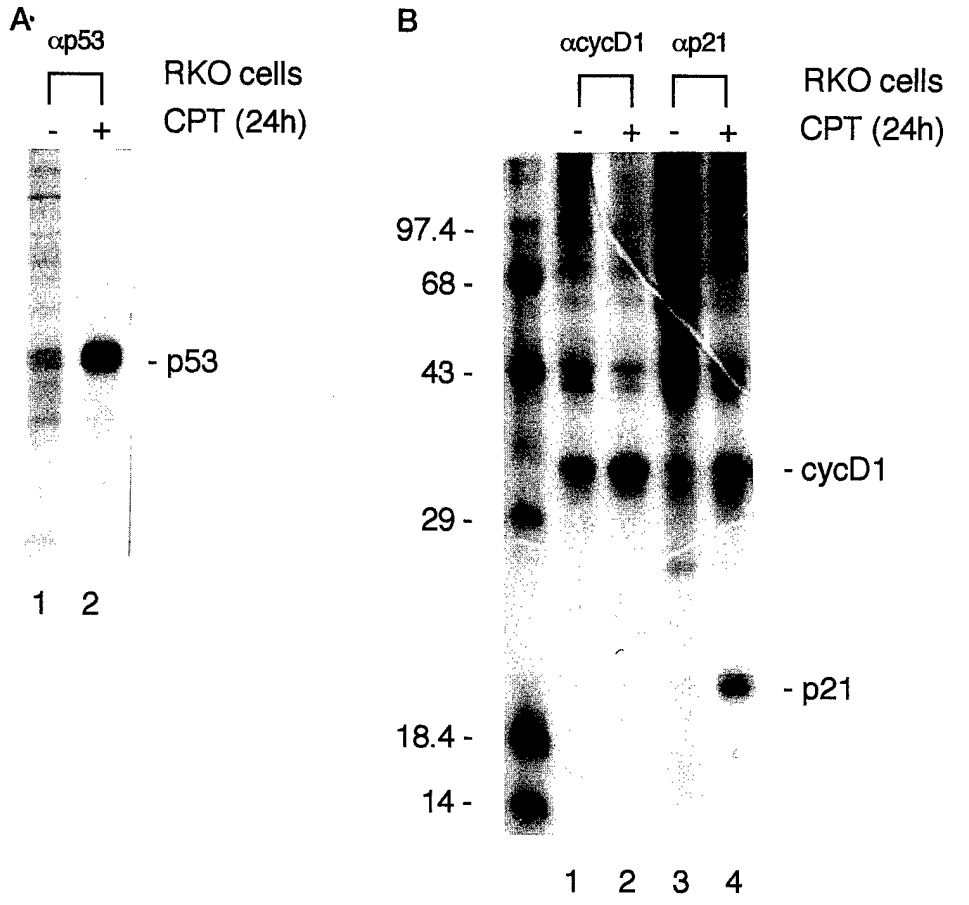

Fig. 4. p53-dependent cyclin D1 induction following DNA damage. ^{35}S-labeled extracts from RKO cells untreated or treated with camptothecin were subjected to immunoprecipitation with anti-p53 antibody PAb1801 (*A*), anti-cyclin D1 (*B*; *Lanes 1* and *2*) and anti-p21 (*B*; *Lanes 3* and *4*) as indicated at the *top* of each lane. *Right,* p53, cyclin D1, and p21 proteins.

conditions. It remains possible that p53 can directly, albeit weakly, activate expression of the cyclin D1 promoter. However, an alternative explanation is that cyclin D1 induction by p53 may be mediated by one or more p53 responsive genes.

p21 Expression Induces Cyclin D1. Since the *p21* gene, itself a target of p53, is involved in cell cycle regulation, we asked whether p21 can mediate the p53-dependent induction of cyclin D1 expression. To this end, the human p21 cDNA was cloned downstream of a cytomegalovirus immediate early gene promoter producing a p21 expression vector (pcDNA3-p21). Following transient transfection with pcDNA3-p21, T98G cells were ^{35}S-labeled, and the amounts of the p21 and cyclin D1 proteins were determined by immunoprecipitation. As expected, with increasing amounts of transfected pcDNA3-p21 DNA, p21 protein was detected in a dose-dependent manner (Fig. 6, *Lanes 6* and *7*), although the highest amount of transfected DNA reduced somewhat the transfection efficiency (Fig. 6, *Lanes 7* and 8). This increase in p21 was commensurate with a significant increase in the amount of the cyclin D1 protein in the transfected cells (Fig. 6, compare *Lane 1* with *Lanes 2–4*). Furthermore, there were markedly greater quantities of cyclin D1 in the p21 immunoprecipitates (Fig. 6, compare *Lane 5* with *Lanes 6–8*). The fact that the cyclin D1 stimulation by p21 was relatively modest compared to what was observed with GM47–23 or 3-4 cells is most likely due to the fact that the efficiency of transient transfection is relatively low (around 5% of total cells). Therefore, it is probable that the induction of cyclin D1 was significantly greater than what we detected. We conclude that p21 mediates the induction of cyclin D1 expression by p53.

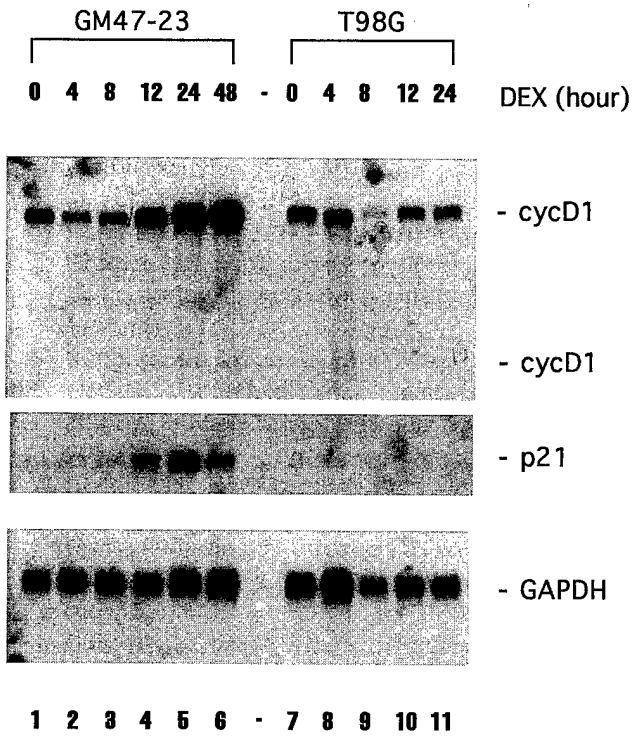

Fig. 5. p53-dependent transcriptional induction of cyclin D1. A Northern blot was prepared using 0.5 μg of poly(A)$^+$ RNA samples isolated either from GM47–23 cells treated with dexamethasone for 0–48 h (*Lanes 1–6*) or from T98G cells treated with dexamethasone for 0–24 h (*Lanes 7–11*) at the times indicated. The blot was probed sequentially with cyclin D1 cDNA (*top panel*), p21 cDNA (*middle panel*), and GAPDH cDNA (*bottom panel*). *Right,* identities of cyclin D1, p21, and GAPDH mRNAs.

Discussion

We have provided evidence that accumulation of the wild-type p53 protein leads to induction of cyclin D1 expression. This induction is

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p53 levels, functional domains, and DNA damage determine the extent of the apoptotic response of tumor cells

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Wagner et al. 1994). Furthermore, different reports have provided essentially contradictory results as to the requirement for the sequence-specific transactivation function of p53 for induction of apoptosis (Haupt et al. 1995; Sabbitini et al. 1995). If indeed there is an alternative function of p53, then it will be important to identify the domains or regions of the protein responsible for this function.

p53 has been subjected to extensive analysis of its functional domains (Gottlieb and Oren 1996; Ko and Prives 1996). The p53 polypeptide consists of an activation domain located within the amino-terminal 43 amino acids, a sequence-specific DNA binding domain located within the central, conserved portion of the protein, and, within the carboxyl terminus resides a tetramerization domain as well as a regulatory region that controls the ability of the protein to allosterically switch from a latent form to one that is active for sequence-specific DNA binding. Either naturally occurring or experimentally produced mutations within these regions of p53 have identified residues that are important for their function. Mutation of two residues within the p53 activation region, leu22 and trp23, abrogates transactivation by p53 (Lin et al. 1994). This is presumably the case because these residues are required for the interaction of the activation domain with TATA-box binding protein associated factors (TAFs) (Lu and Levine 1995; Thut et al. 1995). The vast majority of the missense mutations that have been detected in tumors of cancer patients map to the central DNA-binding domain of p53 (Hollstein et al. 1991). Among these are a number of mutational hot spots that occur with unusually high frequency and together make up ~40% of all tumor-derived p53 mutations. Most mutations within the central DNA-binding domain impair or abolish sequence-specific DNA binding by p53 (Vogelstein and Kinzler 1992). Finally, mutational analysis of the carboxyl terminus has revealed residues that are important for oligomerization of the p53 protein (Sturzbecher et al. 1992). Moreover, deletion of the carboxy-terminal 30 amino acids of p53 has striking stimulatory effects on the ability of the protein to bind to DNA in vitro (Hupp et al. 1992; Halazonetis and Kandil 1993).

To further understand how p53 might regulate arrest versus apoptosis we have used the p53 null cell lines Saos2 and H1299 to generate tetracycline-regulated cell lines inducibly expressing either wild-type or mutant forms of p53 or wild-type p21. This system has been utilized previously by others for a variety of goals (Buckbinder et al. 1994; Van Meir et al. 1994; Agarwal et al. 1995; Chen et al. 1995). The lines we have generated have allowed us to (1) identify a quantitative response to different amounts of p53, (2) examine a number of p53 variants in clonally derived cell populations to derive information about the influence of functional domains of p53 on cell arrest and apoptosis, and (3) determine the effect of DNA damage on p53 in this context. Our results have provided new insight into p53 and should provide a paradigm for this approach to studying p53 structure and function.

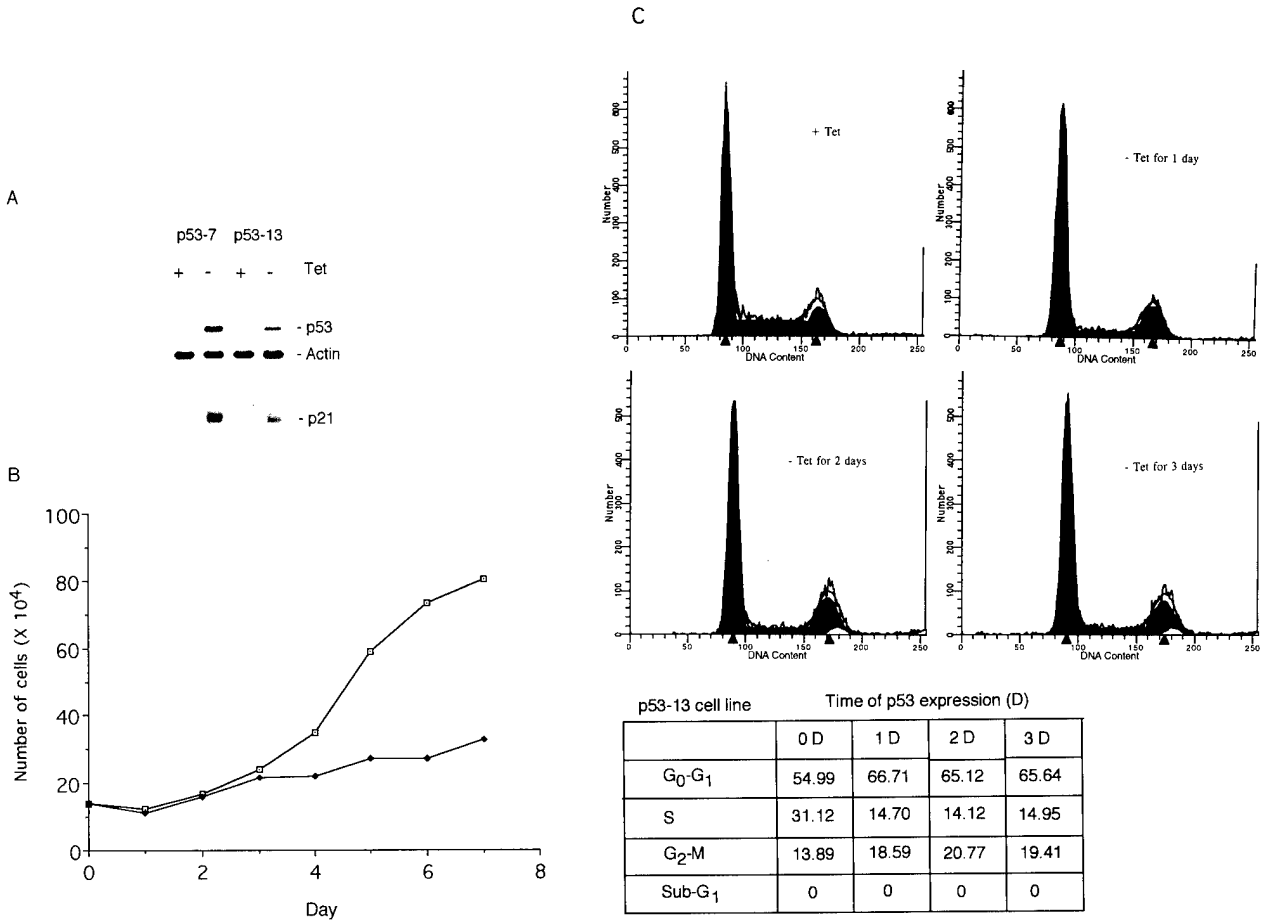
Results

The level of p53 within Saos2 cells determines cell death or arrest

To generate the first series of inducible cell lines, we chose the human osterosarcoma cell line Saos2 because they are null for p53, are easily transfected, and have been shown previously to arrest upon overexpression of wild-type p53 (Chen et al. 1990). Each individual cell line used for this study was chosen from a number of clonal lines based on relative levels of protein expressed after induction.

Of the two wild-type p53 inducible cell lines obtained, one (p53-7) expressed p53 protein at relatively high levels upon withdrawal of tetracycline and will be referred to as the "high p53 producer" line (Fig. 1A). p53 protein was detected within 8 hr and reached maximal levels at 24 hr post-induction (data not shown). Note that the amount of detectable induced p53 protein in p53-7 cells, however, was substantively less than that induced in DNA damaged human RKO cells that contain wild-type p53 (Kastan et al. 1992), demonstrating that the level of p53 expressed in the Saos2 cell line is within the physiological range. When the growth curve of induced and uninduced p53-7 cells was examined, there was a dramatic difference in cell viability between the two states (Figure 1B). The uninduced cells continued to grow with a doubling time of ~48 hr, whereas the p53 expressing cells started to die within 2 days (as determined by reduced cell count), and by 3 days, only 10% of the cells survived. By 5 days after induction there were virtually no visible cells remaining on the plate (data not shown). DNA histogram analysis of induced p53-7 cells (Fig. 1C) showed that 1 day after tetracycline withdrawal the percentage of cells in S phase was reduced from 14% to 5%, and the percentage of cells in G_2 was increased from 14% to 23%, suggesting that a transient G_2 arrest had occurred in at least a fraction of the cells; at this time point little or no sub-G_1 content cells were scored. By 2 days, however, 25% of the cells had a sub-G_1 DNA content and at 3 days after induction >60% of the cells had sub-G_1 DNA content indicating apoptotic death, with the remaining cells primarily arrested in G_1. Virtually all cells die after induction of p53 in p53-7 cells, thus, the arrest must be transient because the cells are not protected from eventually entering the apoptotic pathway. It should be mentioned that upon continued passage of p53-7 cells, the apoptotic phenotype of the cells was diminished and eventually lost. However, the levels of p53 were still high and the cells still exhibited the cell-cycle arrest component of the response.

When the second p53 cell line (p53-13) was analyzed, upon withdrawal of tetracycline, the amount of p53 within p53-13 cells was ~25–50% of that detected in p53-7 cells (Fig. 2A). Although p53-13 cells showed substantially slowed cell growth, there was in contrast with p53-7 cells, no reduction in cell number (Fig. 2B) nor did cells with sub-G_1 DNA content appear (Figure 2C). DNA histogram analysis showed that the percentage of S phase p53-13 cells was reduced from 31% to 15% within



p53-13 cell line — Time of p53 expression (D)

	0 D	1 D	2 D	3 D
G_0-G_1	54.99	66.71	65.12	65.64
S	31.12	14.70	14.12	14.95
G_2-M	13.89	18.59	20.77	19.41
Sub-G_1	0	0	0	0

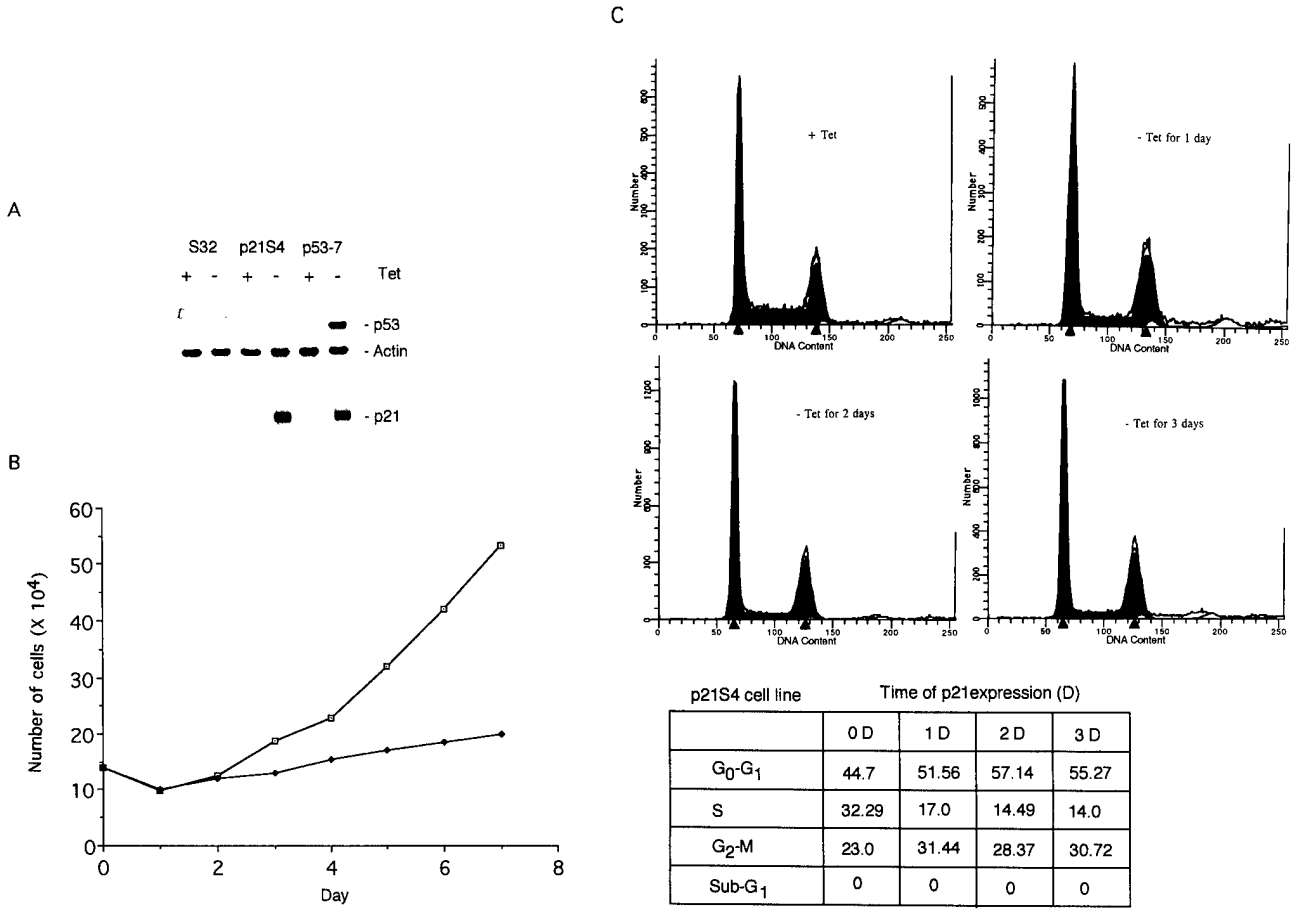
Figure 2. Induction of cell-cycle arrest by low levels of p53 in p53-13 cells. The experiments were performed in an identical manner to those in Fig. 1.

DNA damage can sensitize cells to p53-mediated apoptosis without affecting the level of p53 protein

Effectors of DNA damage have been shown to increase the amount of p53 in cells by a post-transcriptional mechanism (Maltzman and Czyzyk 1984; Kastan et al. 1992; Lu and Lane 1993). Because the quantity of p53 induced was clearly a determinant of the switch between arrest and apoptosis in p53-7 cells, we wished to test whether the levels of p53 in the low producer cell line, p53-13, could be augmented after DNA damage, and whether the cells would now undergo apoptosis. Camptothecin (CPT), a topoisomerase inhibitor and cancer therapy drug, has been shown to induce DNA damage in cells (Nelson and Kastan 1994). Moreover, as shown in Figure 1A, we have confirmed that treament of RKO cells with CPT results in a significant induction of p53 protein levels. When p53-13 cells were treated with increasing amounts of CPT in the presence or absence of tetracycline we observed that even without p53 induction there was a modest apoptotic response to CPT, suggesting that Saos2 cells can undergo DNA damage-associated apoptosis in a p53-independent manner (Fig. 4B). Unexpectedly, however, when p53 was induced in CPT-treated p53-13 cells there was a significant increase in the number of apoptotic cells (Fig. 4B), and yet no discernable increase of p53 protein levels (Fig. 4A). Thus, p53 and CPT cooperate in Saos2 cells to cause a strong apoptotic response, and this occurs in a manner that is independent of p53 protein accumulation.

p21 induction in Saos2 cells leads to arrest but not apoptosis

The cyclin-dependent kinase inhibitor p21 (WAF1), a potential mediator of p53 tumor suppression, has been shown in a number of studies to be strongly induced by p53. Consistent with these results p21 expression was markedly increased in both p53-13 and p53-7 cells after removal of tetracycline (Fig. 2A). Because the low p53 producer cells (p53-13) did not undergo apoptosis this



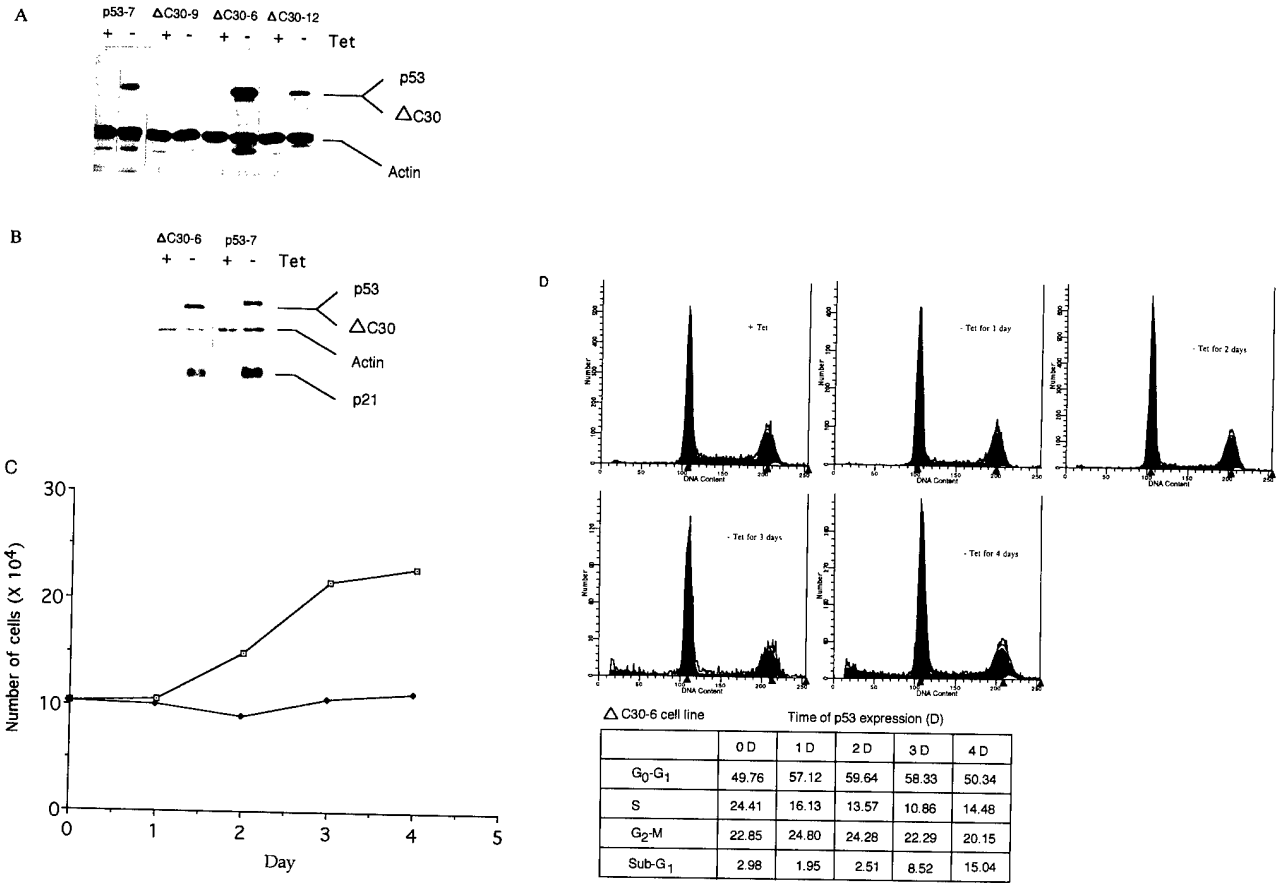
p21S4 cell line	Time of p21expression (D)			
	0 D	1 D	2 D	3 D
G_0-G_1	44.7	51.56	57.14	55.27
S	32.29	17.0	14.49	14.0
G_2-M	23.0	31.44	28.37	30.72
Sub-G_1	0	0	0	0

Figure 5. p21 induction leads to cell cycle arrest but not apoptosis. The experiments were performed as those in Fig. 1.

p53(gln22/ser23) (data not shown). The amount of p53 in 22/23-4 cells was approximately equivalent to that detected in p53-7 cells. Consistent with the lack of induction of p21 in 22/23-4 cells, no detectable cell-cycle arrest was observed after induction of mutant p53 as observed by DNA histogram analysis (Fig. 6). Nevertheless, in these cells p53(gln22/ser23) reproducibly induced cell death (Fig. 6), although to a lesser extent and with delayed kinetics as compared with wild-type p53. Thus, those cells that did not undergo apoptosis contained a normal S-phase DNA content and presumably kept cycling. The apoptosis induced by p53(gln22/ser23), although reduced, was significantly greater than either the background levels of cell death that occur in the presence of tetracycline, or than in cells expressing mutant p53 completely defective in apoptosis (see Fig. 8, below, for comparison). Our data confirm and extend observations by Oren and colleagues (Haupt et al. 1995), who showed that apoptosis can be brought about by p53 mutants such as p53(gln22/ser23) that are defective in sequence-specific transactivation in transiently transfected HeLa cells. These data also provide clear evidence that the abilities of p53 to induce cell-cycle arrest and apoptosis are genetically separable.

The p53 carboxyl terminus is necessary for efficient apoptosis

p53 contains an autoinhibitory region within the last 30 amino acids of the protein. Deletion of this region generates a p53 protein that is activated for DNA binding in vitro (Hupp et al. 1992) and that is comparable to full-length p53 in activating transcription in transient transfection assays in cells (Halazonetis and Kandil 1993; L. Ko, unpubl.). To determine the cellular response to p53 lacking the carboxy-terminal 30 amino acids p53(ΔC30), cell lines expressing this p53 variant were isolated. One of these lines, p53(ΔC30)-6, contained at least twofold more p53 than the high producer cell line p53-7 when normalized to the cellular actin protein levels (Fig. 7A), although still in the range of p53 induced in DNA-damaged RKO cells. Consistent with observations that the ΔC30 mutant is functional in transactivation, p21 was induced in p53(ΔC30) cells to a similar extent as in wild-type p53 cells (Fig. 7B). Although the growth of p53(ΔC30)-6 cells was completely arrested upon induction, the cell number did not decrease detectably throughout the time course of the experiment (Fig. 7C). Because very slow but detectable cell growth had been



Δ C30-6 cell line — Time of p53 expression (D)

	0 D	1 D	2 D	3 D	4 D
G_0-G_1	49.76	57.12	59.64	58.33	50.34
S	24.41	16.13	13.57	10.86	14.48
G_2-M	22.85	24.80	24.28	22.29	20.15
Sub-G_1	2.98	1.95	2.51	8.52	15.04

Figure 7. The carboxyl terminus of p53 is required for full apoptosis. (*A*) Inducible expression of wild-type p53 and p53(ΔC30), and levels of actin in p53-7, p53(ΔC30)-9, p53(ΔC30)-6, and p53(ΔC30)-12 cell lines in the presence or absence of tetracycline (1 μg/ml) for 24 hr. The blot was reacted with p53 monoclonal PAb1801 and actin polyclonal antibodies. (*B*) The inducible expression of wild-type p53, p53 (ΔC30) and p21, and levels of actin in p53-7 and p53(ΔC30)-6 cells in the presence or absence of tetracycline (1 μg/ml) for 24 hr. The blot was reacted with a mixture of p53 monoclonal PAb1801 and actin polyclonal antibodies, and p21 monoclonal antibody, respectively. (*C*) and (*D*) The experiments were performed in an identical manner to those in Fig. 1B and 1C.

arrest. However, the high producer H1299 cells displayed a more rapid and extensive apoptotic response than seen with the Saos2 high producer cell line. The increased kinetics seen with this cell line may be caused by the faster doubling time of H1299 cells (e.g., 24 hr) as compared with Saos2 cells (~48 hr). Interestingly, a cell line with inducible p53 lacking the first 22 amino acids was as potent in inducing apoptosis as wild-type p53. Since p21 accumulated after induction in these cells, this suggests that p53(ΔN22) is transcriptionally active, although whether this truncated p53 is fully comparable to wild-type p53 is not yet established.

Consistent with results observed in Saos2 cells, H1299 lines expressing ser249, his275, or ΔN96 mutant forms of p53 were completely unable to induce apoptosis (Table 1). Moreover, cells expressing the carboxy-terminally truncated mutant p53(ΔC30) or the *trans*-activation-defective mutant p53(gln22/ser23) underwent apoptosis with reduced kinetics and extent over the time course examined. Importantly, a p53 variant that contained both the amino-terminal double mutation at residues 22 and 23 but that also lacked the carboxy-terminal 30 amino acids when expressed at high levels in H1299 cells, was completely inert in inducing apoptosis or growth arrest. This finding provided the strongest evidence that both the amino- and carboxyl terminus of p53 are required for apoptosis in tumor cells.

Discussion

Inducible cell lines provide insight into p53 responses in tumor cells

The cell lines described above have provided several novel observations about the cellular response to p53. We show for the first time that within a given clonal cell line the level of p53 can determine whether cells arrest or die. We also demonstrate that although DNA damage can cooperate with p53 to elicit an apoptotic response, this occurs without detectable alteration in the amount of the p53 protein. Furthermore, our results show that the arrest and apoptotic response are genetically separable activities of p53. Finally, our data suggest that the p53 protein has multiple domains that function in in-

tle p53 is required to drive expression of p21 and consequently to effect growth arrest.

It is likely that p53 transactivation also contributes to the apoptotic response because the cell death induced in response to the transactivation-defective p53 p53(gln22/ser23) occurs with delayed and reduced kinetics as compared with that seen with wild-type p53. p53(ΔC30), which lacks the carboxy-terminal 30-amino-acid regulatory domain but has comparable ability to activate transcription, also induces a weak and delayed apoptosis. However, the doubly altered mutant p53(gln22/ser23ΔC30) is inert for such activity (Table 1). Therefore, our data imply that although the ability of p53(gln22/ser23) to induce apoptosis is *trans*-activation-independent, that of p53(ΔC30) is *trans*-activation-dependent, highlighting the fact that p53 acts to induce apoptosis by at least two discrete pathways.

Because p21 induction is not correlated with p53-mediated apoptosis in the cell lines examined, there may be alternate p53 target genes involved in apoptosis that might be bound relatively weakly by p53 and would thus require more p53 protein to ensure sufficient site occupancy for transcriptional activation. Interestingly, the tumor-derived mutant forms p53(ala143) (Friedlander et al. 1996) and p53 (pro175) (Ludwig et al. 1996) are defective in inducing apoptosis but can induce transcription from a limited subset of p53 responsive elements. These mutants can activate transcription from promoters with responsive elements from p21, mdm-2, and cyclin G, but not Bax or IGFBP3 genes whose cognate sites, notably, are bound relatively poorly by p53. Although the Bax gene is an obvious candidate for an apoptotic p53 target gene, induction of Bax RNA or protein was not observed in Saos2 cells (data not shown), implying that other p53 responsive genes are activated in these cells. We are currently examining whether other candidate target genes such as IGFBP3 are activated by p53 in these inducible cell lines.

The role of p21 in arrest and apoptosis

Cell lines expressing p21 underwent arrest but not apoptosis in the absence of p53. The growth curves and FACS profiles of cells expressing p21 were similar to those expressing lower levels of p53 (cf. Figs. 2 and 5). In each case a dramatically reduced growth rate was accompanied by arrest in both G_1 and G_2. Although we cannot rule out that other targets of p53 may also be involved, these data imply that the arrest response of Saos2 and H1299 cells to moderate levels of p53 is caused primarily by induction of p21. It is also clear that p21 induction in these cells is insufficient to induce apoptosis. This conclusion is derived from the following results: (1) High levels of p21 expressed either with or without p53 did not cause apoptosis in Saos2 cells; (2) the p53(ΔC30) cell line, which shows a very reduced apoptotic response, is as effective as wild-type p53 in inducing p21 and cell cycle arrest; and (3) the transcriptionally defective p53 mutant p53(gln22/ser23) can not induce p21 (nor can it effect a cell-cycle arrest) and yet it can induce apoptosis, albeit to a lesser extent.

Despite the fact that p21 was induced to comparable levels in p21- and p53-inducible cell lines, the high producer p53-7 cells underwent both arrest and apoptosis. The fact that apoptosis was the eventual fate of virtually all p53-7 cells indicates that the p21-mediated arrest in these cells is not sufficient to protect the cells from the death response.

Speculation on a transcription-independent role for p53 in apoptosis

Given data from previous studies as well as the data presented here, it is clear that p53 can induce apoptosis in Saos2 and H1299 cells without transcriptional activation. Clearly, however, in some cases transcriptional activation is required (Sabbatini et al. 1995; Attardi et al. 1996). Although there is as yet a lack of full understanding of the reason for the differences in requirements noted, it can be speculated that species, cell type, and immortalization status differences may be involved. The magnitude of the apoptotic response varied dramatically with the p53 mutant that was induced (Fig. 8 and Table 1). Because the extent and kinetics of apoptosis induced by intact wild-type p53 are far greater than those by either of p53(ΔC30) or p53(gln22/ser23), we propose that transcription-dependent and -independent apoptotic pathways induced by these p53 variants, respectively, cooperate to induce a full apoptotic response. Cells expressing p53(ΔN96), which lacks the amino-terminal 96 amino acids but has an intact carboxyl terminus, can not induce apoptosis. Taken together with the results of the p53(ΔC30) mutant we conclude that both amino- and carboxyl termini must be intact to produce a strong p53 apoptotic response. Again, the fact that a mutant p53 with both a mutated amino terminus and a truncated carboxyl terminus [p53(gln22/ser23ΔC30)] is absolutely inert for both apoptosis and arrest in H1299 cells underscores this conclusion. Paradoxically, however, tumor-derived mutants that contain intact amino and carboxyl termini are also completely inert for inducing apoptosis. It is well established that the one feature common to the tumor-derived p53 mutations is a defect in sequence-specific p53 DNA binding. Therefore, our results suggest that p53 might need to be bound to cognate sites in DNA but not necessarily activating transcription for it to be in the correct conformation for its role in apoptosis. Alternatively, the identification of the cellular proteins 53BP1 and 53BP2 that can bind to the central core region of wild-type but not mutant p53 (Iwabuchi et al. 1994) provides the possibility that there may be cellular proteins rather than DNA with which this region of p53 must associate.

To explain our results, we propose the following model (Fig. 9): Interactions with a bi- or multicomponent factor would be required to associate with regions both at amino and carboxyl termini of p53, when it is bound to DNA, to cause apoptosis. Whereas the amino-termi-

ence or absence of tetracycline. Cells were extracted 24–48 hr later, and expression of the p53 protein was determined by Western blot analysis. Two of the 15 clonal Saos2 cell lines (S32 and S2) and two of the 30 clonal H1299 cell lines (H15 and H24) were found to induce p53 expression upon withdrawal of tetracycline. Both S32 and H24 cell lines were used as parental cell lines for subsequent generation of inducible cell lines on the basis of their lower basal (leaky) expression of the tTA transactivation. Second, various 10-3 plasmids containing cDNAs encoding either wild-type or mutant forms of p53 or p21 were cotransfected with the puromycin selectable pBabe plasmid into either S32 or H24 cells. Clones were selected and maintained in the presence of 2 μg and 1 μg of puromycin (Sigma) per ml, respectively. Individual clones were screened for inducible expression of the p53 and p21 proteins by Western blot analysis using monoclonal antibodies against p53 and p21 as described below.

Immunoblot analysis

Cells were collected from plates in PBS, resuspended with 1× sample buffer, and boiled for 5 min. For immunoblot analysis, a standard procedure was followed as described previously (Chen et al. 1995. Monoclonal antibodies PAb1801 and PAb421 were used to detect p53. The affinity-purified monoclonal antibodies against p21 (Ab-1) and Bcl-2 (Ab-1) were purchased from Oncogene Science (Uniondale, NY) and affinity-purified monoclonal antibodies against Bax (P-19) and antiactin polyclonal antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Sigma (St. Louis, MO), respectively.

Growth rate and cell-cycle analyses

To determine the rate of cell growth, 1×10^5 cells were seeded per 60-mm plate with or without tetracycline. The medium was replaced with fresh medium with or without tetracycline every 48 hr. At indicated times, cells were trypsinized and collected individually from at least two plates. Cells from each plate were counted with a hemacytometer at least twice. The average number of cells from at least two plates were used for growth rate determination.

For cell-cycle analysis, 2.5×10^5 cells were seeded per 90-mm plate with or without tetracycline. The medium was replaced every 48 hr as needed with fresh medium with or without tetracycline. At the indicated times, cell were trypsinized and fixed with 2 ml of 70% ethanol for at least 30 min. For FACS analysis, the fixed cells were centrifuged and resuspended in 1 ml of PBS solution containing 50 μg/ml each of RNase A (Sigma) and propidium iodide (PI) (Sigma). The stained cells were analyzed in a fluorescence-activated cell sorter (FACSCaliber, Becton Dickinson) within 4 hr. The percentage of cells in various cell cycle phases was determined by using the CellFit program.

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